

Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate *in vivo* melanoma tumor growth

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Abstract. Phenothiazines and related antipsychotics were reported to have an antiproliferative effect in several tissue cultures. The aims of this study were: a) to screen *in vitro*, the potential anti-cancer activity of phenothiazines in wild-type and multi-drug resistant (MDR) B16 mouse melanoma cell lines; and b) to determine the *in vivo* anti-tumor effect of an *in vitro* selected highly potent phenothiazine (thioridazine) in a murine melanoma model. The following phenothiazines were evaluated: perphenazine, fluphenazine, thioridazine, trifluoperazine and chlorpromazine. All agents induced a dose-dependent decrease in cell viability in wild-type and in MDR B16 melanoma cells. Thioridazine displayed the highest antiproliferative activity. Flow cytometric analyses of 24-h treated B16 melanoma cells revealed an increase in fragmented DNA (16.3 vs 71.3% and 87.2% in controls, 25 μ M and 50 μ M thioridazine-treated, respectively). Apoptosis was confirmed by co-staining of thioridazine-treated B16 cells (12.5 μ M) with propidium iodide and Hoechst 33342 reagents. Caspase-3 expression, a typical mediator of apoptosis, was markedly increased following a 4-h exposure of B16 cells to thioridazine (25 μ M and 50 μ M). This increase could be blocked by a specific caspase-3 inhibitor. *In vivo* studies were performed using female C57/Bl mice. Animals were inoculated with wild-type B16 cells by i.v. injection into the tail vein. Mice were treated with thioridazine (10 and 15 mg/kg x3/week i.p. or 15, and 25 mg/kg/day p.o.) and control animals received saline. Mice were monitored for 21-30 days. Body weight was recorded. After autopsy, the lung weight and number of pulmonary melanoma colonies were determined. Thioridazine administration (i.p. or p.o.) resulted in the reduction of lung

tumor burden and an increase in mice survival. In conclusion, several phenothiazines, and particularly thioridazine, induced apoptosis of B16 melanoma cells and demonstrated *in vivo* anti-tumor activity.

Introduction

Some psychotropic agents belonging to various classes, including antipsychotics, antidepressants and mood stabilizers, have been described to possess a significant *in vitro* antiproliferative activity (1). There are conflicting results concerning cancer epidemiology in schizophrenia. However, most studies demonstrate a low prevalence of melanoma in schizophrenia patients, an effect that could be attributed to neuroleptic treatment (2,3).

Phenothiazines have been shown to exert antiproliferative activity in a concentration-dependent manner, in a variety of tumor cell lines, such as HL-60, and H33-HJ JA1 leukemia (4) and IRSC-10M small-cell lung cancer cells (5). Other reports revealed that haloperidol and the phenothiazines, fluphenazine and flupenthixol, induced marked dose-dependent cell death in neuroblastoma and glioma cell lines (6-8).

We have previously reported that some antipsychotics induce apoptosis in human neuroblastomas (SK-N-SH, and SH-SY5Y) and rat C6 glioma cells and, to a much lower degree, in primary mouse brain tissue (9-11). When the effect of different antipsychotics (typical and atypical) was examined in SH-SY5Y cells, the phenothiazine, perphenazine, was found to be the most potent antiproliferative agent, which arrested the cell cycle at the G1 phase (11). Perphenazine and fluphenazine were also reported to arrest human MCF-7 breast cancer cells at the G1 phase of the cell cycle and to reduce the percentage of cells in the S phase (12). Although the *in vitro* effect of different psychotropics on cell proliferation and differentiation has been extensively studied, there is scarce data on the *in vivo* efficacy of these agents in animal models of cancer.

Patel *et al* (13) showed that the phenothiazine, chlorpromazine, administered subcutaneously caused a marked reduction in the rate of [³H]thymidine incorporation into the brain DNA of juvenile rats. In accordance with these data, Kola and Folbs (14) reported that chlorpromazine inhibited the mitotic index of mouse blastocytes.

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The main goal of the present study was to select a phenothiazine with potent *in vitro* anti-tumor activity for *in vivo* evaluation in a murine melanoma model.

Materials and methods

Reagents for tissue culture. Minimum essential medium (MEM), horse serum, fetal calf serum (FCS), glucose, glutamine, gentamycin, Leibowitch medium L-15, DCCM, RPMI and trypsin were obtained from Beith Haemek, Israel. Neutral red reagent, perphenazine, chlorpromazine, thioridazine, fluphenazine, trifluoperazine, propidium iodide (PI) and Hoechst reagent were from Sigma, St. Louis, MO, USA. Substrate (Ac-DEVD-AMC) was from Biomol Plymouth Meeting, PA, USA, and caspase-3 inhibitor (Ac-DEVD-CHO) was from Alexis, Lausen, Switzerland. Thioridazine was dissolved in saline and the other phenothiazines were dissolved in lactic acid (1%) and further diluted in phosphate-buffered saline (PBS).

Cell lines. Mouse melanoma (B16), and mouse colchicine resistant B16 melanoma, found to possess multi-drug resistant (MDR) properties (15) were used. Mouse B16 melanoma cells (both types) were maintained in RPMI-1640. Media was supplemented with 10% heat-inactivated FCS, penicillin (100 μ /ml), streptomycin (100 μ g/ml), nystatin (12.5 μ /ml), and L-glutamine (2 mM). Confluent cultures were washed with PBS, detached with trypsin (0.25%), centrifuged, and subcultured in 96-well microtiter test plates.

Cell viability. The cell viability was measured using neutral red staining (16). Absorption of neutral red by lysosomes causes coloring of living cells. Quantitative analysis was performed by colorimetric assay (ELISA reader at 550 nm). Viability was tested in cells treated with phenothiazines compared to vehicle-treated cells (saline and lactic acid 0.01-0.05%) which did not modify basal cell viability. Results were expressed as % of controls (vehicle treated cells).

Flow cytometry of cells. Fragmentation of cell nuclei was studied by flow cytometric analysis of propidium iodide (PI)-stained mouse B16 cells, as previously described (17). Cells were treated with thioridazine (12.5, 25, and 50 μ M) for 24 h. Analysis was performed using a fluorescence activated cell sorter (FACS; Becton-Dickinson, Mountain View, CA) equipped with an argon ion laser adjusted to an excitation wavelength of 480 nm and with a doublet discrimination module (DDM). Lysis II (Becton-Dickinson) software was used for data acquisition and determination of apoptotic nuclear changes.

Fluorescence of Hoechst plus PI-stained B16 melanoma. The cells were co-stained with DNA binding dyes, Hoechst 33342 (20 μ g/ml) and PI (10 μ g/ml), for 5 min at room temperature, 24 h after exposure to thioridazine 12.5 μ M or saline. The cells were then examined using a fluorescence microscope with ultraviolet excitation at 340-380 nm. Intact nuclei of control cells were detected by typical blue Hoechst fluorescence, while cells undergoing apoptosis were detected by fragmented nuclear red PI fluorescence (18).

Caspase-3 activity. Caspase-3 activity was measured by an enzymatic fluorimetric method using a fluorogenic substrate (Ac-DEVD-AMC) producing blue fluorescence detected at a 360-nm wavelength (19). AMC was cleaved from the substrate by caspase-3 (and caspase-3-like enzymes) and produced a yellow-green fluorescence monitored by fluorimeter at 460 nm. The amount of yellow green fluorescence is proportional to the activity of caspase-3 in the cell extract sample. B16 cell lysates were prepared by Triton X-100 extraction 4 h after exposure to thioridazine (25 and 50 μ M) as well as positive controls of serum-free medium and vehicle treated control cells. Whole cell lysates were added to a buffer containing 100 μ M peptide substrate, 100 mM HEPES, 10% glycerol, 1 mM EDTA and 10 mM dithiothreitol. Measurements were determined every 10 min for 150 min. The reaction was also monitored in the presence of specific caspase-3 inhibitor, DEVD-AMC-CHO (19), which was added after 60 min to the reaction mixture to ascertain the specificity of the enzyme.

Animal studies. Female C57/Bl mice at 5-7 weeks of age were purchased from Harlan, Jerusalem, Israel. The animals were housed (6 or 7/cage) under controlled conditions (temperature, light, humidity) and given food and water *ad libitum*. After 7 days of acclimatization, the animals were inoculated with B16 melanoma cells by i.v. injection (200,000 cells/mouse) into the tail vein.

Ethics. The study was approved by the Institutional Ethical Committee for Animal Experimentation at Rabin Medical Center, Petah-Tiqva, Israel. Principles of Laboratory Animal Care were followed (see NIH publication No. 85-23, revised 1985).

Intra-peritoneal administration. Female C57/Bl mice at 5-7 weeks of age were used. The mice were inoculated with B16 melanoma cells and divided randomly (7/cage) into 4 treatment groups (14 mice each): a) healthy untreated mice; b) B16 inoculated, saline treated; c) B16 inoculated, thioridazine (10 mg/kg) treated; and d) B16 inoculated, thioridazine (15 mg/kg) treated.

The treatment was initiated 3 days after cell inoculation (drug or saline was injected i.p. 3 times weekly in the morning). Half of the animals were sacrificed after 21 days, and half after 27 days. The lung weight and number of lung metastases were recorded.

Administration via oral route. Female C57/Bl mice at 5-7 weeks of age (total of 54 animals) were used. The mice were inoculated with melanoma B16 cells and divided randomly (6/cage) into 3 treatment groups: a) B16 inoculated, saline treated; b) thioridazine treated (15 mg/kg/day); and c) thioridazine treated (25 mg/kg/day). Thioridazine was administered daily in the morning using a feeding needle, starting on day 3 after cell inoculation. The body weight was registered 3 times weekly. The animals were sacrificed after 23, 27, and 30 days (1/3 of animals each time), and the survival rate, lung weight, and lung metastasis number were recorded.

Statistics. Cell viability and proliferation were expressed as mean $\pm$ SEM of 3-4 determinations for each assay. The

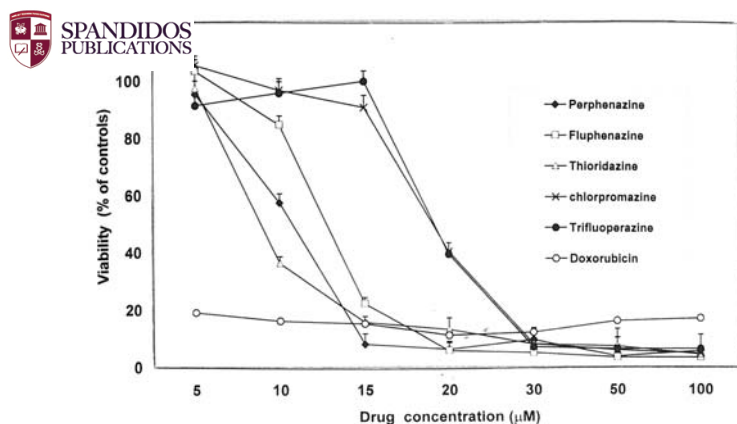


Figure 1. Concentration-dependent effect of various phenothiazines and doxorubicin (5-100 μ M) on viability (neutral red) of wild-type B16 mouse melanoma cells 24 h after exposure to drug. Each point represents mean $\pm$ SEM of 4 determinations.

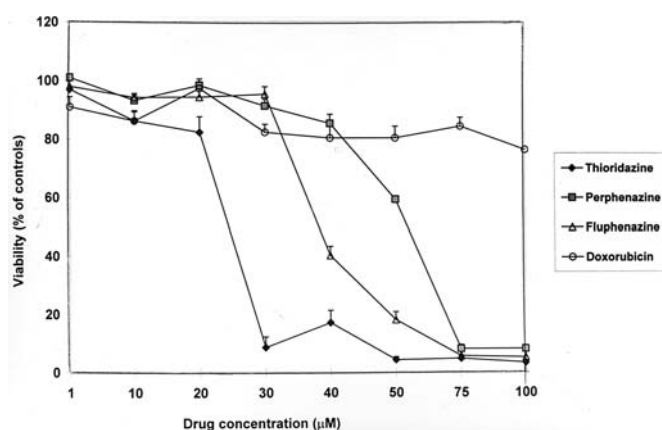


Figure 2. Concentration-dependent effect of various phenothiazines and doxorubicin (1-100 μ M) on viability (neutral red) of MDR B16 mouse melanoma cells 24 h after exposure to drug. Each point represents mean $\pm$ SEM of 4 determinations.

difference between thioridazine and vehicle treated cells or animals was determined using the unpaired Student's t-test and P-values of <0.05 were considered statistically significant.

Results

In vitro studies. The effect of different phenothiazines as compared to an established anti-tumor agent, namely doxorubicin, at a concentration range of 5-100 μ M is shown in Fig 1. All phenothiazines (thioridazine, perphenazine, fluphenazine, trifluoperazine and chlorpromazine) induced a dose-dependent inhibition of cell viability up to 95% of basal (IC_{50} values were 10.9, 11.6, 13.4, 14.6 and 36.6 μ M for the drugs listed above, respectively). Doxorubicin was found to be highly active, inducing 81% inhibition of cell viability at the lowest concentration of 5 μ M. When the experiment was conducted in MDR B16 melanoma (Fig. 2), resistance to doxorubicin was evidenced with only 24% inhibition of viability up to a doxorubicin concentration of 100 μ M. In contrast to doxorubicin, a dose-dependent inhibition of cell viability (up to 95%) was observed using the phenothiazines,

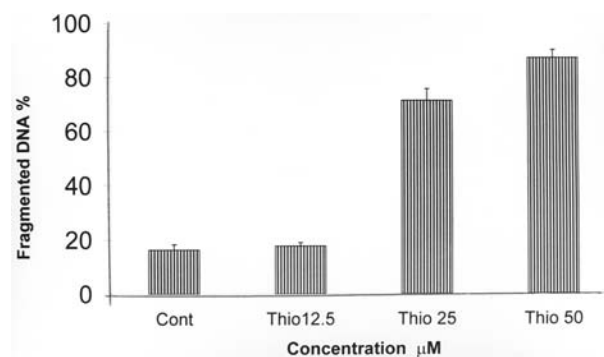


Figure 3. Effect of thioridazine 12.5, 25 and 50 μ M on DNA fragmentation of PI-stained B16 melanoma cells evaluated by flow cytometry. Each point represents % of fragmented DNA and is the mean of three determinations using data analysis of Lysis II (Becton-Dickinson) software.

thioridazine, perphenazine and fluphenazine, although the IC_{50} levels of the phenothiazines were also 3- to 4-fold higher than those found in the wild-type B16 melanoma cell line (28.2, 40.1 and 56.9 μ M for thioridazine, fluphenazine and perphenazine, respectively). With the aim of characterizing the phenothiazine-induced cell death mechanism, we evaluated the effect of the most potent agent, thioridazine, on the fragmentation of nuclei of wild-type B16 melanoma cells using FACS analysis and nuclear staining with PI 24 h after exposure to the drug. As shown in Fig. 3, thioridazine (25 μ M and 50 μ M) caused a marked increase in DNA fragmentation (71 and 87% as compared to 16% in controls). A lower concentration of 12.5 μ M of thioridazine was ineffective compared to control cells.

Fig. 4 is a microscopic photograph of wild-type B16 melanoma cells double-stained with PI and Hoechst and exposed to vehicle or thioridazine (12.5 μ M). The photograph demonstrates a large number of red (PI) fluorescence of fragmented nuclei in treated cells compared to blue (Hoechst) fluorescence of intact nuclei treated with vehicle. Fig. 5 demonstrates caspase-3 activity measured for 2 h alone, and in the presence of the caspase-3 inhibitor, following 4-h exposure of wild-type B16 cells to thioridazine or to saline. The data show that caspase-3 activity rose during 2 h of measurements, it was markedly stimulated (3.3- and 12.8-fold compared to vehicle treated control cells by thioridazine at 25 μ M and 50 μ M respectively). We further showed that the induced enzymatic stimulation was blocked by the addition of specific caspase-3 inhibitor, DEVD-AMC-CHO.

In vivo studies

Intraperitoneal route. Follow-up of animals until the 21st day after B16 cell inoculation resulted in no difference in lung weight between healthy, untreated and thioridazine-treated B16 inoculated mice. In the 4th week post inoculation, there was a rapid deterioration in the health of the cancerous animals. The healthy mice showed no significant change in body weight but the untreated B16 mice exhibited a sharp decrease in body weight (mean, -2.65 g), the thioridazine-treated (10 mg/kg, i.p.) mice decreased in weight by 1.5 g, and the thioridazine (15 mg/kg) increased weight by 0.6 g (Fig. 7). With regard to lung weight (Fig. 6), on day 21 there was no significant difference in lung weight between the healthy and the cancer

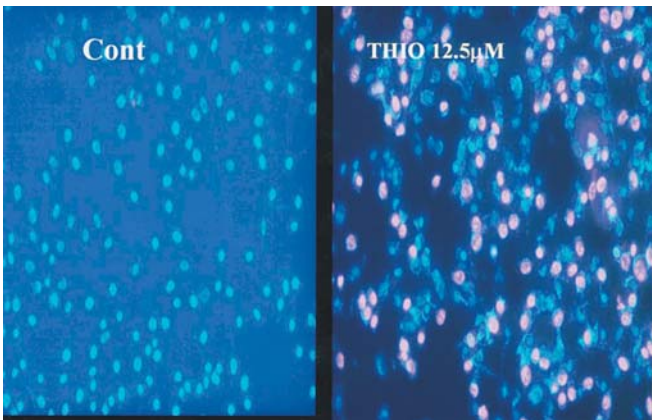


Figure 4. Fluorescence microscopy picture of nuclei of B16 melanoma cells treated with thioridazine 12.5 μ M or vehicle and stained with PI + Hoechst. Fragmented reddish stain represents cells undergoing apoptosis.

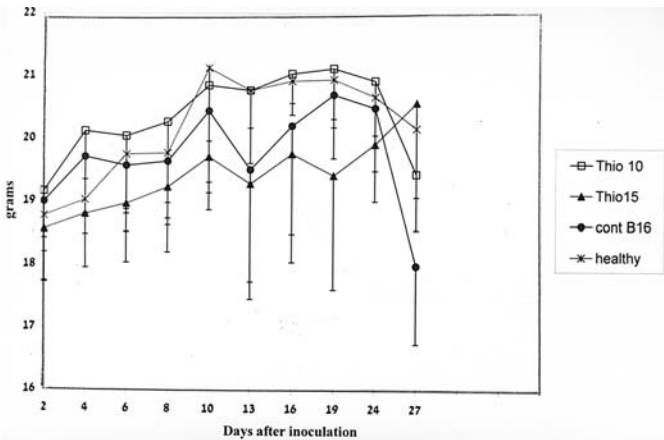


Figure 7. Body weight curve in healthy controls, B16, and B16 inoculated mice treated i.p. with thioridazine (10, 15 mg/kg x3/week). Each point is the mean $\pm$ SEM of 7 animals.

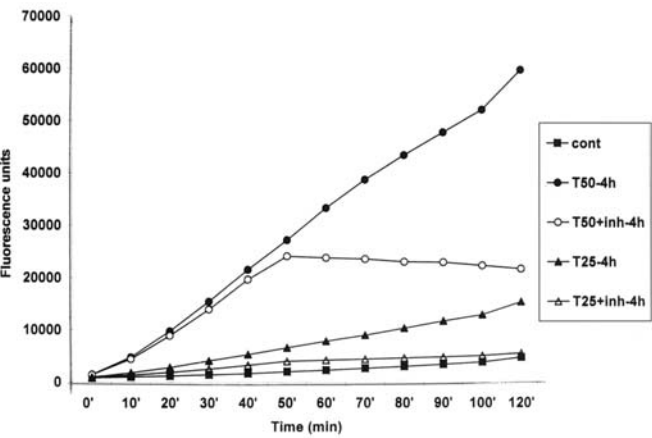


Figure 5. Caspase-3 activity measured by enzymatic fluorimetric method in B16 melanoma cells exposed for 4 h to serum-free (SF) conditions or to thioridazine (T) at concentrations of 25 and 50 μ M alone or combined with caspase-3 inhibitor (inh), DEVD-AMC-CHO. Samples were assessed every 10 min for 2 h. Each point is the mean of 2 determinations.

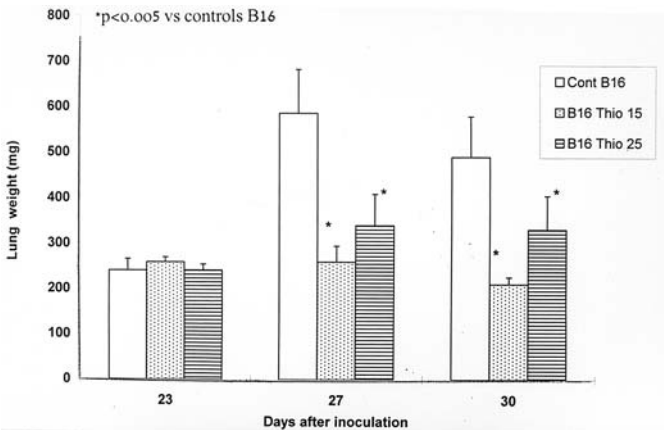


Figure 8. Lung weight of C57Bl female mice inoculated with B16 melanoma cells and treated by oral administration with thioridazine (15 or 25 mg/kg/day) or vehicle and sacrificed on day 23, 27 or 30 after cell inoculation. Each column represents the mean $\pm$ SEM of 7 determinations.

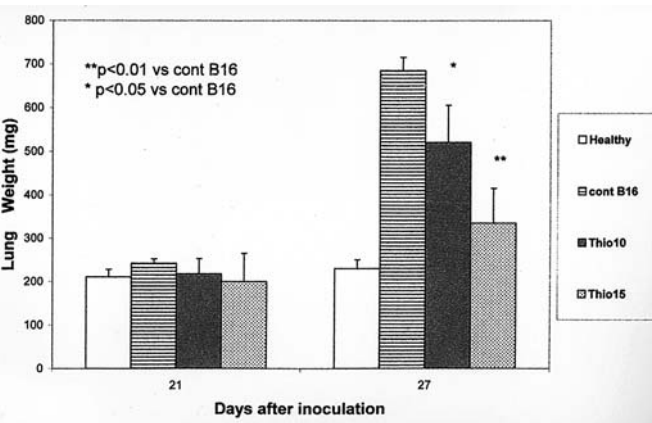


Figure 6. Lung weight of C57Bl female mice inoculated with B16 melanoma cells and treated with ip administration of thioridazine (10 or 15 mg/kg/ x3/week) or vehicle and sacrificed on day 21 or 27 after cell inoculation. Each column represents the mean $\pm$ SEM of 7 mice.

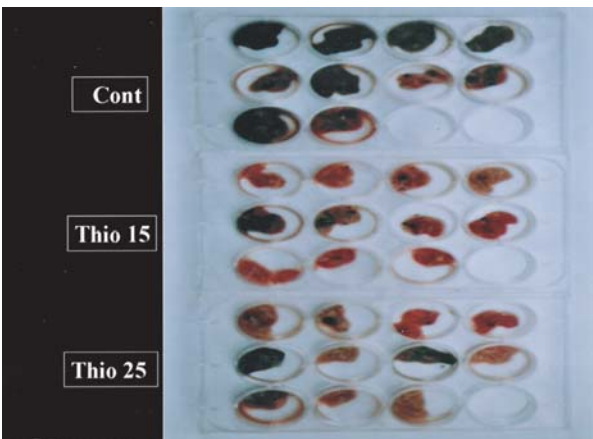


Figure 9. Picture of mice lungs inoculated with B16 melanoma cells and treated by oral administration with vehicle, thioridazine 15 mg/kg or 25 mg/kg/day.

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exposed to all the treatment regimens. However, in 1 in the control B16 inoculated group, a confluence of tumor colonies (>250) in the lungs was observed, whereas only a few colonies were detected in the 6 remaining mice. In the thioridazine-treated mice, we did not find confluence of tumor colonies, and the total number of colonies in the lungs was significantly lower (vehicle: 53.8 ± 11.4 vs. 46.13 ± 7.4 and 28.5 ± 3.4 for thioridazine 10 mg/kg and 15 mg/kg, respectively; ($p < 0.05$ controls vs Thio 15). When mice were sacrificed on the 27th day after inoculation, a marked increase in lung weight was found in all the cancerous groups; the increase was significantly attenuated by thioridazine treatment. An inverse relationship ($r = -0.97$) was found between thioridazine dose and lung weight. The difference between lung weight in the thioridazine groups and the control animals was significant ($p < 0.05$ and $p < 0.01$ for thioridazine 10 and 15 mg/kg, respectively). Vehicle treated B16 inoculated animals presented confluent lungs (6/7), thioridazine (10 mg/kg) also showed confluence in (6/7) mice, and thioridazine (15 mg/kg) showed confluence only in 2/7 mice.

Oral route. Follow-up of animals until the 23rd day after B16 cell inoculation showed that there was no difference in lung weight between the B16 control group and the thioridazine-treated groups. In the next autopsies on the 27th and 30th days of the experiment, a marked difference was found between the B16 control mice and the thioridazine-treated groups (Fig. 8). Confluent metastases were found on days 27-30 (pooled data) in 8/10 B16 control animals and in 4/22 in thioridazine-treated groups (another 2 animals died before the 27th day in both the controls and the treated mice groups, and their lungs were not collected) (Fig. 9 picture). A small size and low number of metastases were found also in thioridazine-treated animals. Spontaneous death during experiment occurred in 5/18 B16 control group and in 2/36 thioridazine-treated mice. No difference was found in the body weight of healthy mice, B16 controls and thioridazine treated mice.

Discussion

Our data provide evidence that several phenothiazines, mainly thioridazine, effectively inhibited proliferation of mouse melanoma cells both in wild-type as well as in MDR cells via proapoptotic mechanism. These results support ours and others' previous data showing that several neuroleptics induce apoptosis in neuroblastoma and glioma cells and in several other cancer cell lines (4-7,10,11). We found that, among the different phenothiazines, thioridazine had the most potent antiproliferative activity in both wild-type and MDR cells, followed by perphenazine and fluphenazine, while chlorpromazine and trifluoperazine were less efficacious. Our data showed that the effect of thioridazine was accompanied by the stimulation of apoptotic activity mediated by enzyme caspase-3 activation which, in turn, was inhibited by the specific caspase-3 inhibitor (Ac-DEVD-CHO). This pathway generally represents typical apoptosis-related alterations in mitochondrial function, including release of cytochrome C, which precedes caspase activation (19-21). Pronounced efficacy in causing apoptosis in the B16 cells was achieved at a concentration of $12.5 \mu\text{M}$ thioridazine, as shown by the high

percentage of reddish PI-stained nuclei in the fluorescence microscopic picture of PI + Hoechst-stained cells, whereas the sensitivity of the flow cytometry analysis was somewhat lower, resulting in a significant increase in the fragmented DNA only at higher concentrations. The difference between the two methods can be related to a difference in method sensitivity. Similar results were reported with thioridazine in rat glioma C6 and human neuroblastoma cell lines (10). In wild-type cells, the chemotherapeutic agent, doxorubicin, was more efficacious compared to the phenothiazines, causing 81% inhibition of cell viability at the concentration of $5 \mu\text{M}$ as compared to IC_{50} of 10-20 μM of phenothiazines. In contrast, in the MDR cells, up to 100 μM doxorubicin was not efficacious and thioridazine, perphenazine and fluphenazine induced a dose-dependent inhibition of cell viability, although with an IC_{50} concentration which was 2-3 times higher than that found in wild-type cells.

Several studies showed that phenothiazines and their derivatives sensitized MDR cells to the effect of chemotherapeutic agents, an effect that might be mediated by increasing the concentration of the chemotherapeutic agent in the cells (22). One of the major obstacles in inducing responsiveness in cancer cells is the existence of efflux transporter, P-glycoprotein (Pgp), which is linked to multi-drug resistance (MDR) genes. The inhibitory effect of phenothiazine derivatives on Pgp was demonstrated and correlated with the drug's hydrophobicity (23). Furthermore, thioridazine was found to reverse doxorubicin-resistance in sarcoma 180 cells by blocking Pgp (24). In the present study, we showed that phenothiazines were also efficacious in cell growth suppression by direct proapoptotic effect as well as by circumventing the Pgp efflux transporter.

Based on the *in vitro* results, we performed *in vivo* studies and selected thioridazine as a lead agent. Thioridazine, a phenothiazine neuroleptic, was until recently commonly prescribed for schizophrenia and related disorders because it produces relatively less frequent motor side effects. In recent years, its use was somewhat limited due to cardiovascular toxicity and QTc prolongation (25). Our results using thioridazine administered both via i.p. and p.o. routes showed that this antipsychotic agent effectively inhibited tumor growth as reflected by the inhibition of the increase in lung weight and the number of pulmonary melanoma colonies. In the i.p. experiment, thioridazine exerted an antiproliferative effect at 10-15 mg/kg while doses >30 mg/kg were found to be toxic (data not shown). In the orally administered thioridazine experiment, the efficacious dose was in the range of 15-25 mg/kg. At these doses, no apparent undesirable side-effects were noted. Interestingly, in terms of lung weight in the orally administered thioridazine, the lower dose of 15 mg/kg was more efficacious compared to the 25 mg/kg. This difference could be explained by the general toxicity of the higher dose. Supporting this possibility is the study of Petruska *et al* (26) who reported a high mortality rate on the administration of the phenothiazine, chlorpromazine hydrochloride, at doses >20 mg/kg in mice. In another study designed to evaluate the potential chemopreventive and anti-tumor effect of phenothiazines *in vivo*, Azuine *et al* (27) showed that several phenothiazines, particularly phenoxazine, effectively inhibited tumor growth in mouse carcinogenic models.

Finally, our results suggest that several phenothiazines, particularly thioridazine, may be useful as chemopreventive or anticancer agents in the treatment of melanoma and particularly of resistant states. Phenothiazines were also indicated for cancer patients due to their role in the symptomatic management of agitation, delirium, psychotic symptoms and nausea (28). It seems that selected phenothiazines might be of value in the armamentum of cancer therapy as an addition to established chemotherapeutic agents.

Acknowledgments

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