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ORIGINAL ARTICLE

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Phenothiazines suppress proliferation and induce apoptosis in cultured leukemic cells without any influence on the viability of normal lymphocytes

Phenothiazines and leukemia

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Abstract *Purpose*: The purpose of the present study was to investigate the effects of phenothiazines (at clinically relevant doses) on the viability and proliferation of leukemic cell lines and normal lymphocytes, and to investigate the possibility of specific induction of apoptosis in leukemic cells. *Methods*: Phenothiazines with different chemical structure and hydrophobicity were used: chlorpromazine (CPZ); levomepromazine (LVPZ); prometazine (PMZ); trifluoperazine (TFPZ); thioridazine (TRDZ). The leukemic cell lines used were: Daudi and Raji (derived from Burkitt's lymphoma), K-562 (derived from myelogenous leukemia), and BALL-1, MOLT-4, HPB-ALL and CCRF-HSB-2 (derived from acute lymphoblastic leukemia). The cytotoxicity of the phenothiazines was determined by a CellTiter-Glo luminescent cell viability assay, using ATP bioluminescence as a marker of cell viability as well as a marker of mitochondrial activity. The proliferation of leukemic cells was determined using a CellTiter-AQ cell proliferation assay which is based on the reduction of a methyl-tetrazolium compound to the formazan product. Apoptosis induction was estimated using phosphatidylserine (PSer) translocation to the cell surface and DNA fragmentation as characteristics of the process. *Results*: Phenothiazines (at concentrations in the range 0.1–10 μM) did not affect the viability of normal lymphocytes during a 24-h incubation. Moreover, about 15–20% increase in ATP bioluminescence was observed in normal cells during treatment with 40 μM phenothiazines. In contrast, the phenothiazines manifested strong cytotoxicity and antiproliferative activity against leukemic cells. The most powerful drugs were TFPZ and TRDZ, followed by CPZ. They showed a significant cytotoxic effect against leukemic cells even at 5–10 μM. The most sensitive cell lines were MOLT-4 and Raji, and the most resistant were HPB-ALL and CCRF-HSB-2. All phenothiazines induced PSer exposure on the surface of leukemic cells, but not of normal lymphocytes. TFPZ, TRDZ and CPZ also induced DNA fragmentation in almost all leukemic cell lines during a 48-h incubation. The strongest apoptotic agent was TRDZ. The apoptosis induction was not accompanied by a significant release of cytochrome *c* from the mitochondria into the cytoplasm of native cells. Moreover, the drugs markedly suppressed Ca^{2+}-induced cytochrome *c* release in isolated mitochondria of leukemic cells. *Conclusions*: The results suggest that in clinically relevant doses (up to 20 μM) some phenothiazines (TFPZ, TRDZ, CPZ) expressed a selective cytotoxicity and antiproliferative activity, and induced apoptosis in leukemic cells without any influence on the viability of normal lymphocytes. It is considered that the mechanism of apoptosis induction in phenothiazine-treated leukemic cells is associated with inhibition of mitochondrial DNA polymerase and decreased ATP production, which are crucial events for the viability of cancer cells.

Keywords Cultured leukemic cells · Normal lymphocytes · Phenothiazines · Cytotoxicity · Proliferation · Apoptosis

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Abbreviations *CPZ* Chlorpromazine · *LVPZ* Levomepromazine · *PBS(−)* Phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) · *PMZ* Prometazine · *PSer* Phosphatidylserine · *ROS* Reactive oxygen species · *TFPZ* Trifluoperazine · *TRDZ* Thioridazine

Introduction

Cancer is a stressful life event, and patients differ in their ability to cope with it. Some patients demonstrate psychological disturbances, such as depression, agitation, anxiety, psychosis, and acute confusional state [1, 2]. Sometimes psychotropic drugs are indicated to treat these behavioral changes or to control the major side effects of chemotherapy. In contrast to the broad data available on the beneficial effects of these agents on the psychiatric complications of cancer, little is known about their effect on carcinogenesis and progression of the cancer itself. It is essential to determine whether these relatively inexpensive and widely available drugs or their derivatives could serve as an important addition to the armamentarium of cancer therapy.

The aim of the present study was to clarify the effects of some psychotropic drugs of the phenothiazine family on cancer cell viability and proliferation in leukemia. Phenothiazines are interesting also for their photosensitizing effects and potential for use in photodynamic therapy of cancer [3, 4, 5, 6]. In human carcinoma cells, decreased efflux of conventional chemotherapeutic agents on exposure to phenothiazines has been suggested to be responsible for the reversal of such cells from drug resistance to drug sensitivity, which is a clinical challenge [4, 7, 8].

A growing body of evidence, mainly from in vitro studies, indicates that phenothiazines have an inhibitory effect on normal and cancer cell growth and differentiation [6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. However, this activity usually is achieved at high pharmacological concentrations, and the relevance to clinically relevant therapeutic doses remains unclear. Many authors accept that phenothiazines are cytostatic, but not cytotoxic [4, 18, 19, 20]. The cytotoxicity of phenothiazines is a disputable question and it is not clear whether phenothiazines influence the viability and homeostasis of normal cells at doses used for the treatment of behavioral changes in cancer patients. To our knowledge, this is the first study in which the dose-dependent effects of phenothiazines on the viability of leukemic cells and normal lymphocytes have been investigated in parallel, and demonstrating that the drugs (at clinically relevant doses) express selective cytotoxicity and antiproliferative activity against cancer cells without any influence on the viability of normal lymphocytes. The experiments were also designed to investigate the development of apoptosis, using PSer translocation to the cell surface, cytochrome *c* release from mitochondria into the cytoplasm, and DNA fragmentation as characteristics of the process.

Materials and methods

Reagents

All reagents were of analytical grade and were obtained from Amersham Pharmacia Biotech (Piscataway, N.J.), Becton Dickinson (Franklin Lakes, N.J.), Gibco BRL (Invitrogen, Carlsbad, Calif.), Seikagaku Company (Tokyo, Japan), Sigma Chemical Company (St. Louis, Mo.), and Wako Pure Chemical Industries (Osaka, Japan). Phenothiazines were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)]. Their final concentrations in the samples are given in the figure legends.

Preparation of cells

The following human leukemic cell lines were used in these experiments: Raji and Daudi (derived from Burkitt's lymphoma); K-562 (derived from myelogenous leukemia); and BALL-1, HPB-ALL, MOLT-4, CCRF-HSB-2 (derived from acute lymphoblastic leukemia). The cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37°C. The cell lines were generous gift from Dr. J. Minowada (Hayashibara Biochemical Laboratories, Okayama, Japan). Normal lymphocytes were purified from heparinized peripheral blood obtained from normal adults (aged 38–40 years) by Lymphosepar I. The cells used for assay were in logarithmic phase. They were sedimented by centrifugation (1000 rpm, 10 min) and washed three times with PBS(−) before the experiments.

Measurement of cytotoxicity

The cytotoxicity of phenothiazines against normal and leukemic cells was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, Wis.), based on ATP bioluminescence as a marker of cell viability [21]. The test was also used for estimation of mitochondrial activity. Briefly, the phenothiazines were dissolved in PBS(−) and dispensed in 10-μl aliquots into 96-well microplates. The cells were suspended in the culture medium to a concentration 5×10^5 cells/ml and then added in 90-μl aliquots to each patch. After different times of incubation at 37°C in a cell incubator under an atmosphere containing 5% CO_2, the CellTiter-Glo kit (Promega) was added in aliquots of 100 μl to each patch and was incubated with the cell suspensions for 1 h, following the procedure recommended by the manufacturer. The luminescence produced by the luciferase-catalyzed luciferin plus ATP reaction was detected using a MicroLumat LB96P multiwell scanning spectrophotometer (EG&G Berthold, Bad Wildbad, Germany). The data were normalized to the control group. The cytotoxicity of phenothiazines was calculated as the percentage decrease in luminescence in comparison with the control. Eight independent experiments were carried out for each phenothiazine concentration, one experiment for each cell line, and two parallel samples for each phenothiazine concentration. In preliminary experiments we established that the cytotoxic effects of phenothiazines increased between 2 and 24 h incubation with the drugs and reached a plateau after 24 h. In further experiments we selected 24 h and 48 h of incubation as the interval allowing the maximal effects of the phenothiazines on the parameters analyzed to be determined, as well as the long-lasting effects of the drugs and the potential for reversibility of their cytotoxicity to be clarified.

Cell proliferation assay

The cells were cultured in the absence and in the presence of phenothiazines (10 and 20 μ*M*). After 24 h of incubation, aliquots of the cell suspensions were analyzed for cell proliferation index using a CellTiter AQ cell proliferation assay kit (Promega). The method was

based on detection of the number of viable proliferating cells. A tetrazolium compound (MTS) was bioreduced by the viable cells into a formazan product in the presence of an electron coupling reagent (phenazine methosulfate), according to the method of Mosmann [22]. The absorbance of the formazan product at 490 nm was recorded in a Immunomini NJ-2300 96-well plate reader (InterMed, Tokyo, Japan). The cell proliferation index of phenothiazine-treated cells was calculated as a percentage in relation to the untreated control cells. Six independent experiments were carried out for each phenothiazine concentration, one experiment for each cell line, and two parallel samples for each phenothiazine concentration.

PSer assay

For the determination of PSer an annexin V-FITC kit (Beckman Coulter) was used. The cells ($5x10^5$ cells/well) were cultured as described above in the absence or in the presence of phenothiazine (10 μ*M*). After 24 and 48 h of incubation, the cells were collected by centrifugation (1500 rpm, 10 min) and washed twice with annexin-binding buffer (PBS containing 2.5 m*M* $CaCl_2$). Cell suspension (100 μl) was incubated with 5 μl FITC-conjugated annexin V (FITC-annexin V) for 10 min at room temperature (about 22°C) in the dark. The cells were washed twice with annexin-binding buffer and resuspended in 100 μl of the same buffer. FITC-annexin V bound to PSer on the cell surface was detected spectrofluorimetrically at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 488$ nm) using a Fluoromark fluorescent microplate reader (Bio-Rad, Hercules, Calif.) [23]. Four independent experiments were carried out for each phenothiazine concentration, one experiment for each cell line, and two parallel samples for each phenothiazine concentration.

DNA fragmentation assay

The cells (5×10^5 cells/well) were cultured as described above in the absence or in the presence of phenothiazine (10 μ*M*). After 24 and 48 h of incubation, the cells were collected by centrifugation (1500 rpm, 10 min). Cell lysis and DNA extraction were achieved using a commercially available kit for DNA fragmentation (apoptosis ladder detection kit; Wako, Japan) based on the method of Ioannou and Chen [24]. The extracted DNA was analyzed by gel electrophoresis using TBE buffer and stained with SYBRGreen I. DNA from apoptotic cells forms a ladder on the gel, while DNA from nonapoptotic cells appears as a single band or smeared on the gel.

The degree of DNA fragmentation was estimated in terms of the number of ladder bands, and was graded (Fig. 3B) as follows: + + + marked formation of ladder bands after 24 and 48 h incubation (i.e. DNA fragmentation present); + + poor formation of ladder bands after 24 h incubation and marked formation after 48 h incubation (i.e. DNA fragmentation present); + no formation of ladder bands after 24 h incubation and poor formation after 48 h incubation (i.e. DNA fragmentation absent at 24 h but present at 48 h); − no formation of ladder bands up to 48 h (i.e. DNA fragmentation absent).

Four independent experiments were carried out, one experiment for each cell line and one sample for each phenothiazine concentration.

Immunoblot analysis of cytochrome *c* release from mitochondria into cytosol of native cells

The cells (Raji, MOLT-4, CCRF-HSB-2 at $5x10^6$ cells/ml) treated with phenothiazines (TFPZ, TRDZ or CPZ at 10 μ*M*) for 24 h were resuspended in isolation buffer (150 m*M* sucrose, 10 m*M* HEPES, 1 mg/ml bovine serum albumin, 0.5 m*M* K-EDTA, 0.5 m*M* K-EGTA, 1 μl/ml protease inhibitor cocktail; pH 7.4; Sigma) and homogenized by 50 strokes in a Dounce homogenizer. Cell lysates were centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant (containing cytosol and microsomal fraction) was used for the determination of cytochrome *c* in the cytoplasm. The supernatant was dissolved 1:1 in 2× Laemmli sample buffer (1.1 *M* Tris-HCl, pH 6.0, 3.3% SDS, 22% glycerol, 10% β-mercaptoethanol, 0.001% bromphenol blue). Samples (containing 60 μg protein) were applied to 5% stacking, 4–12% resolving SDS-polyacrylamide gel. Electrophoresis was carried out in two steps: at 80 V for 15 min, then 120 V for 2 h at room temperature. BioRad Kaleidoskop protein standards were also applied for comparison. After electrophoresis the separated protein fractions were transferred to a Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, N.J.) using an XCell II Blot Module (Invitrogen, Carlsbad, Calif.). The transfer was carried out at 35 V for 18 h at 4°C. A double antibody procedure was used to detect the proteins. The membranes were incubated at room temperature for 1 h with anti-cytochrome *c* (mouse, PharMingen, 1:1000) or anti-α-tubulin monoclonal antibodies (mouse, 1:20,000) as a control. The antibody solution was removed and the membranes were washed three times with PBS containing 0.1% Tween-20 (PBS-T). The membranes were then incubated (1 h at room temperature under agitation) with horseradish peroxidase-conjugated goat anti-mouse IgM (Sigma, 1:5000). Antigen-antibody complexes were visualized by enhanced chemiluminescence using an ECL Advance Western blotting detection kit (Amersham Bioscience).

Isolation of mitochondria

The cells (Raji, MOLT-4, CCRF-HSB-2 at $5x10^6$ cells/ml) were resuspended in ice-cold isolation buffer (150 m*M* sucrose, 10 m*M* HEPES, 1 mg/ml bovine serum albumin, 0.5 m*M* K-EDTA, 0.5 m*M* K-EGTA, 1 μl/ml protease inhibitor cocktail; pH 7.4; Sigma) and homogenized by 50 strokes in a Dounce homogenizer. The mitochondrial fraction was isolated from cell lysates by differential centrifugation and was resuspended in a 250 m*M* sucrose-containing reaction buffer (25 m*M* KCl, 5 m*M* Tris-phosphate, 1 μ*M* K-EDTA, 10 m*M* Trizma base; pH 7.4; Sigma) for Ca^{2+}-dependent induction of cytochrome *c* release and subsequent immunoblotting or in polymerase assay buffer (catalogue no. 1669885; Roche, Indianapolis, Ind.) for assay of DNA polymerase activity using a Teflon homogenizer. Fresh mitochondria were used in all analyses.

Immunoblot analysis of Ca^{2+}-induced cytochrome *c* release from isolated mitochondria

Mitochondrial suspension (2 mg protein/ml) was incubated with 100 μ*M* $CaCl_2$ at 28°C for 15 min. Mitochondria were pelleted by centrifugation at 20,000 *g* for 2 min. The supernatant was subjected to 10% SDS-polyacrylamide gel electrophoresis and immunoblot analysis of cytochrome *c* as described above.

DNA polymerase activity assay

Cellular and mitochondrial DNA polymerase activity was determined in phenothiazine-treated and untreated cells using a DNA polymerase assay kit (Roche, catalogue no. 1669885) with slight modification, as described in the manufacturer's instructions. In brief, leukemic cells were cultured in the absence or in the presence of 10 μ*M* phenothiazine for 24 h. Protein extracts from cells or mitochondrial fraction were isolated according to the method of Weisshart and Knopf [25] and aliquots were used for determination of DNA polymerase activity. The test was based on the ability of DNA-dependent DNA polymerases to incorporate modified nucleotides (digoxigenin- and biotin-labeled) into freshly synthesized DNA during 1 h of incubation. The reaction was stopped by the addition of 0.05 *M* EDTA. Detection and quantification of the synthesized DNA as a measure of DNA polymerase activity followed a sandwich ELISA protocol, as follows. Biotin-labeled DNA was bound to the surface of a streptavidin-precoated microtiter plate module. In the next step, an anti-digoxigenin antibody, conjugated with peroxidase, was added and bound to the digoxigenin-labeled nucleotides. In the final step, the peroxidase substrate was added. The peroxidase catalyzed the cleavage of the substrate, resulting in the production of a colored product. The

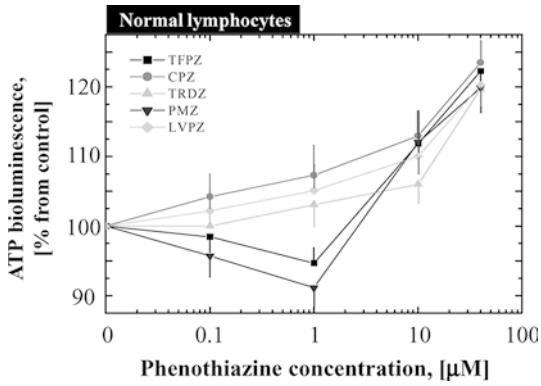

Fig. 1 Effects of phenothiazines on the ATP bioluminescence of normal lymphocytes (during 24 h of incubation) calculated as percentage increase/decrease in ATP bioluminescence in comparison with a control group (luminescence in the absence of phenothiazine; considered as 100%). The results are presented as the means ± SD from eight independent experiments

absorbance of the product, determined at 405 nm using an Immunomini NJ-2300 plate reader (InterMed), was directly correlated with the level of DNA polymerase activity in the sample. The data were normalized against protein concentration in cellular or mitochondrial extracts. All procedures were carried out in nuclease-free conditions. Protein extracts, pretreated with 100 m*M* ammonium sulfate, which is known to suppress eukaryotic DNA polymerases, were used as negative controls.

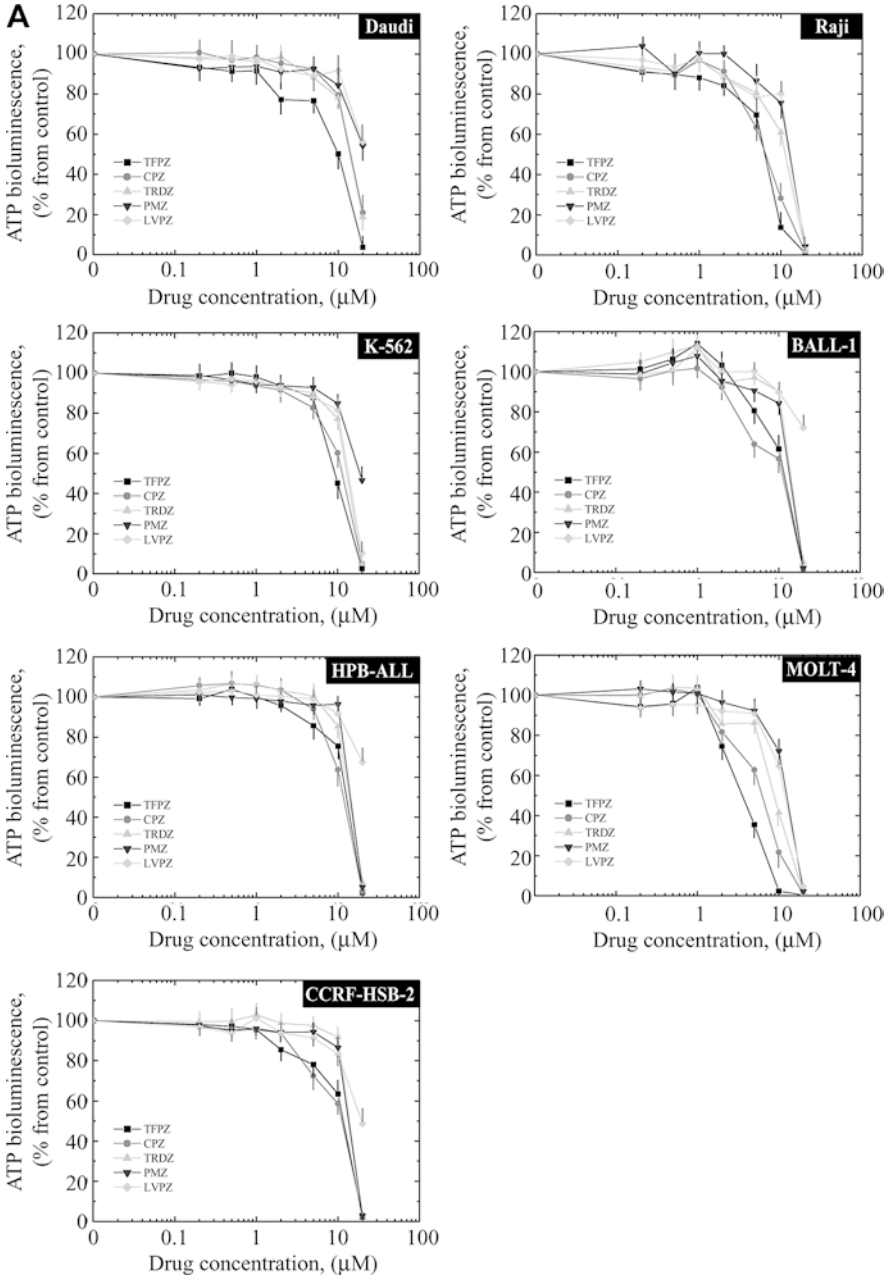

Fig. 2 A Effects of phenothiazines on the ATP bioluminescence of leukemic cells (during 24 h of incubation) calculated as percentage decrease in ATP bioluminescence in comparison with a control group (luminescence in the absence of phenothiazine; considered as 100%). The results are presented as the means ± SD from eight independent experiments. **B** The effects of phenothiazines on cell proliferation calculated as percentage decrease/increase in the number of cells in comparison with a control group (cells grown in the absence of phenothiazine; considered as 100% proliferative activity). The results are presented as the means ± SD from six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs the control group

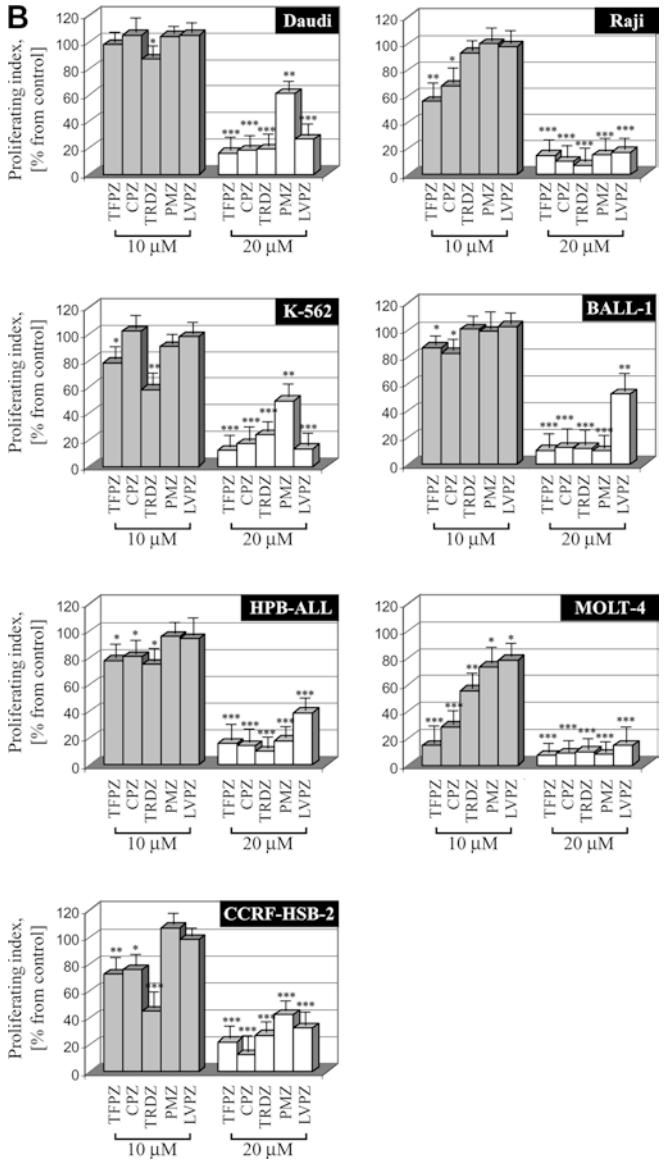

Fig. 2 (Contd.)

Statistical analysis

One-way analysis of variance (ANOVA) was employed, followed by Bonferroni's test for truly significant differences. Statistical significance was defined at $P < 0.05$. The statistical procedures were performed with GraphPad InStat software, version 2.04 (San Diego, Calif.). Data are presented as means ± SD.

Results

The cytotoxicity of phenothiazines was estimated using ATP bioluminescence as a marker of cell viability. The cell viability was calculated as the ratio (in percentage) between ATP bioluminescence in the presence and that in the absence of phenothiazine. As shown in Fig. 1, the phenothiazines did not affect the viability of normal lymphocytes at concentrations in the range 0.1–10 μ*M* during 24 h of incubation. Moreover, an increase of about 15–20% in ATP bioluminescence in the presence of 40 μ*M* phenothiazine was observed in normal cells ($P < 0.05$), which is also an indirect indicator of increased mitochondrial activity.

In contrast, the phenothiazines manifested strong cytotoxic effects against leukemic cells at 20 μ*M* (Fig. 2A). TFPZ and CPZ showed significant cytotoxicity even at 5–10 μ*M*. Calculation of CI_{50} values demonstrated that the most powerful cytotoxic agents were TFPZ and CPZ, followed by TRDZ, PMZ and LVPZ (Table 1). The cytotoxic effects also depended on the cell line and increased in the following order: HPB-ALL < CCRF-HSB-2 < BALL-1 < K-562 = Daudi < Raji < MOLT-4. The most sensitive cells were MOLT-4 and Raji, and the most resistant to phenothiazines were HPB-ALL and CCRF-HSB-2.

Similar effects on the proliferation of leukemic cells in the presence of high concentrations of phenothiazine (10 and 20 μ*M*, Fig. 2B) were seen. At 20 μ*M*, all phenothiazines markedly suppressed leukemic cell proliferation, and TFPZ, CPZ and TRDZ were the strongest inhibitors. However, at 10 μ*M* phenothiazines showed poor (TFPZ, CPZ, TRDZ) or negligible (PMZ, LVPZ) antiproliferative activity against all cell lines except MOLT-4. All the phenothiazines tested showed a well-defined inhibitory effect on the proliferation of MOLT-4 cells.

Proceeding from the fact that drug concentrations about 10 μ*M* correspond to clinically relevant doses (0.4–5.6 ng/ml plasma [26, 27]), we investigated the possibility of induction of apoptosis in leukemic cells and normal lymphocytes treated with 10 μ*M* phenothiazines for 24 and 48 h (Fig. 3). Apoptosis was characterized by PSer exposure on the cell surface as an early apoptotic event, and DNA fragmentation as a last phase. PSer translocation was detected using the FITC-annexin V test. The fluorescence of annexin V-PSer

Table 1 Phenothiazine concentrations causing a 50% decrease in cell viability (CI_{50}). The cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2

Cell line origin	Cell line	CI_{50} (μmol/l)				
		TFPZ	CPZ	TRDZ	PMZ	LVPZ
Normal lymphocytes		> 40	> 40	> 40	> 40	> 40
Burkitt's lymphoma	Raji	6.59	6.95	11.95	13.46	13.90
	Daudi	10.00	14.89	15.30	22.45	23.67
Chronic myelogenous leukemia	K562	9.49	12.04	13.88	14.57	19.18
B-acute lymphoblastic leukemia	BALL-1	11.90	11.19	15.00	14.28	31.71
T-acute lymphoblastic leukemia	MOLT-4	3.57	6.57	9.09	13.37	12.57
	CCRF-HSB-2	12.38	11.81	15.02	14.71	20.00
	HPB-ALL	13.57	12.33	14.76	15.24	28.57

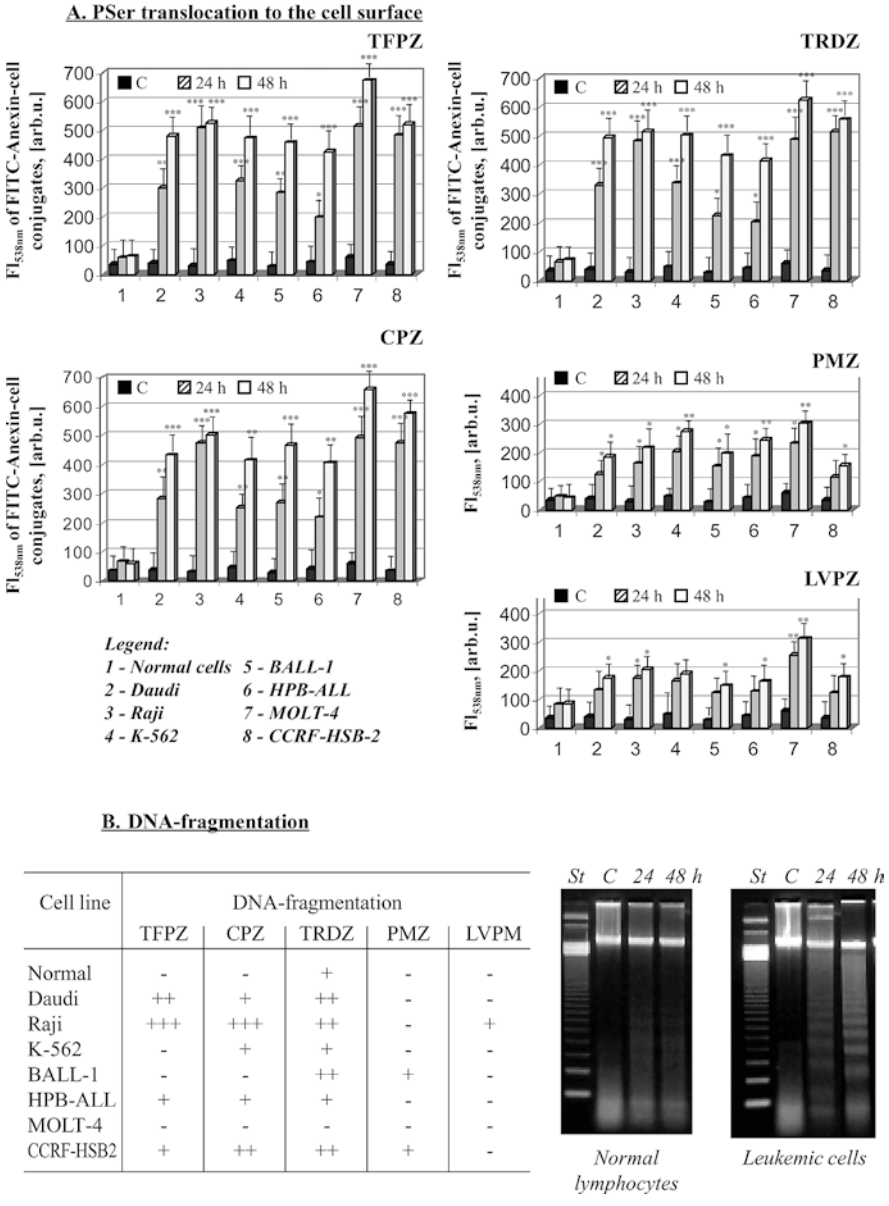

Fig. 3A, B Induction of apoptosis in phenothiazine-treated leukemic cells. **A** PSer exposure on the cell surface. Leukemic cells were cultured in the absence (control *C*) and in the presence of 10 μ*M* phenothiazine for 24 and 48 h. FITC-annexin V bound to cell-surface PSer was detected spectrofluorimetrically. The results are presented as the means ± SD from four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, vs the control group. **B** DNA fragmentation. The cells were cultured in the absence (control *C*) or in the presence of 10 μ*M* phenothiazine for 24 and 48 h. DNA was extracted and DNA fragmentation was detected by gel electrophoresis (*St* standard marker, *C* control untreated cells, *24 h* and *48 h* cells treated with phenothiazine). The degree of DNA fragmentation was estimated in terms of the number of ladder bands and is presented in terms of the grading system described in Materials and methods. Representative blots are from one typical experiment

Cell line	DNA-fragmentation				
	TFPZ	CPZ	TRDZ	PMZ	LVPM
Normal	-	-	+	-	-
Daudi	++	+	++	-	-
Raji	+++	+++	++	-	+
K-562	-	+	+	-	-
BALL-1	-	-	++	+	-
HPB-ALL	+	+	+	-	-
MOLT-4	-	-	-	-	-
CCRF-HSB2	+	++	++	+	-

complexes on the outer monolayer of the plasmatic membrane was used as a measure of the PSer concentration on the cell surface. Phenothiazines did not affect PSer concentration on the cell surface of normal lymphocytes, while in leukemic cells they induced PSer in the outer monolayer. The concentration of PSer increased or gone to a plateau with time of incubation up to 48 h. The values of PSer-annexin V complexes were about two to three times higher after treatment with TFPZ, CPZ and TRDZ than after treatment with PMZ or LVPZ. The most sensitive cell lines were MOLT-4, Raji and Daudi. TFPZ, PMZ and TRDZ also induced DNA fragmentation in all leukemic cell lines except MOLT-4 after 48 h of incubation. DNA from apoptotic cells formed a ladder on the agarose gel, while DNA from nonapoptotic cells appeared as a single band or smeared on the gel. TRDZ was the strongest apoptotic agent of the phenothiazines tested, even in normal lymphocytes. PMZ and LVPZ did not affect DNA (no fragmentation was detected).

Discussion

The phenothiazines (at concentrations corresponding to the clinically relevant plasma concentrations 2–36 μ*M* [26, 27]) markedly decreased the viability of leukemic cells without any cytotoxic effect on normal lymphocytes. Moreover, the ATP bioluminescence of normal lymphocytes increased with increasing drug concentrations up to 40 μ*M*. This finding suggests that including phenothiazines or their derivatives in the treatment of leukemia patients may be clinically beneficial. Clearly, the drugs are promising not only in the treatment of behavioral changes in cancer patients, but also because they have the potential to increase the effect of

conventional antileukemia drugs. Recently, promising preclinical results of phase I and phase II trials of TFPZ and bleomycin have been reported, revealing activity in non-Hodgkin's lymphoma. The combination of the two drugs has been shown to produce greater than expected DNA damage in comparison with the effect observed with either drug alone [17]. It has also been established that phenothiazines potentiate the cytotoxicity of other conventional cancer chemotherapeutics [4, 15, 28].

The cytotoxic effects of phenothiazines were influenced by modification of the drug structure. Substitutions on the phenothiazine ring that increase hydrophobicity (for example, –Cl, $–SCH_3$, and $–CF_3$ groups) lead to more potent cytotoxic effects. The structure-activity relationships of the phenothiazines investigated suggest that the drug with the best-expressed cytotoxic effect against leukemic cells has a hydrophobic nucleus with a $–CF_3$ substitution at position 2 (Fig. 2A). Similar findings have been reported by Molnar et al. [29] in blast-transformed mononuclear cells.

It was established that phenothiazines possessed not only cytotoxic, but also antiproliferative activity against leukemic cells (Fig. 2B). However, strong suppression of cell proliferation was detected at a drug concentration of 20 μM, which is about the upper limit of clinically relevant doses. The most powerful antiproliferative agents were TFPZ and TRDZ, followed by CPZ. In some cell lines (Raji, K-562, MOLT-4, CCRF-HSB-2) they showed a very well-defined antiproliferative effect even at 10 μM. Presumably, the long-term treatment of leukemic cells (more than 24 h) can potentiate the antiproliferative and cytotoxic activity of low doses of TFPZ, CPZ and TRDZ (< 10 μM), as well as of less-effective PMZ and LVPZ.

This assumption was confirmed by the findings concerning apoptosis induction in leukemic cells treated with phenothiazines. Despite the fact that 10 μM TFPZ, CPZ and TRDZ showed low cytotoxicity and antiproliferative activity in leukemic cells with 24 h of incubation, their potential to induce apoptotic cell death increased with increasing time of incubation (up to 48 h). It is of considerable interest clinically that the drugs did not induce apoptotic events in normal lymphocytes. The specific mechanism of induction of apoptosis in leukemic cells by phenothiazines is of substantial interest, because the general safety of the drugs for normal cells and tolerability in cancer patients make further investigation into their use alone or in combination with conventional antileukemia agents justified.

It is widely accepted that phenothiazines are localized predominantly in the mitochondria of normal and cancer eukaryotic cells [30, 31, 32], which is indicative that the mechanism(s) of their cytotoxic activity is probably associated predominantly with mitochondrial function. Since phenothiazines affect ATP production by different ways in normal and leukemic cells (Figs. 1 and 2A), it may be speculated that mitochondria mediate this selectivity. Mitochondria have been shown to play a major role in apoptosis [31, 33, 34]. Moreover, these organelles have a central position in the control of cell

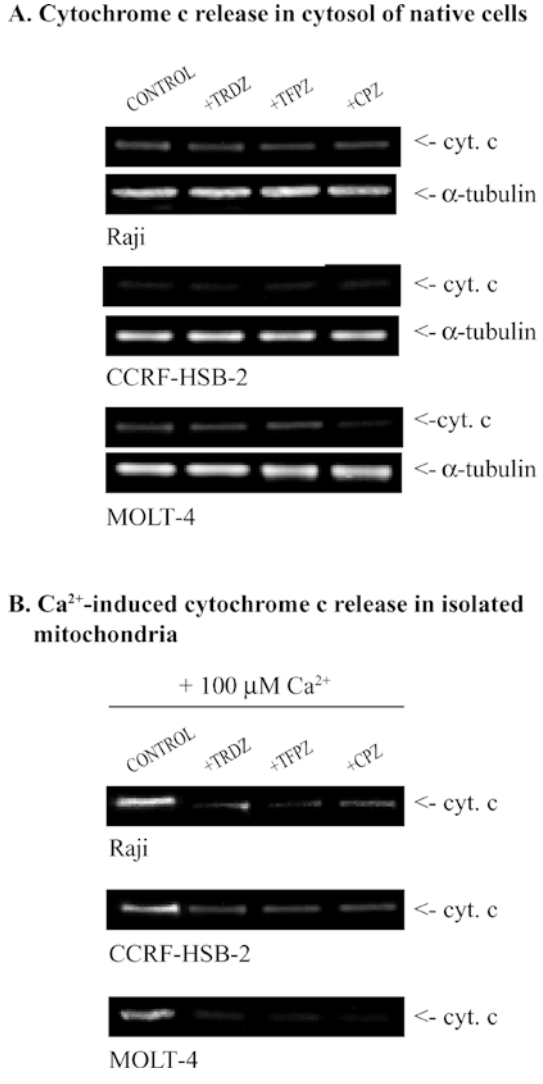

Fig. 4 **A** Effect of phenothiazines on the release of cytochrome *c* from mitochondria into the cytoplasm of native leukemic cells—immunoblot analysis. Leukemic cells (Raji, CCRF-HSB-2 or MOLT-4) were treated with 10 μM phenothiazine for 24 h and cytochrome *c* was determined in the cytosol by Western blotting. Blots from one representative experiment are shown. **B** Effect of phenothiazines on Ca^{2+}-induced cytochrome *c* release from isolated mitochondria—immunoblot analysis. Mitochondria were isolated from phenothiazine-treated or untreated cells as described in Materials and methods. After exposure to 100 μM Ca^{2+} for 15 min, mitochondria were sedimented by centrifugation and cytochrome *c* was determined in the supernatant by Western blotting. Blots from one representative experiment are shown

survival, because they are necessary for the generation of energy required for cell proliferation. Mitochondria consume large amounts of molecular oxygen for ATP synthesis. However, continued consumption of oxygen by mitochondria routinely leads to the generation of different types of ROS [31]. Such oxidants can induce apoptosis if not detoxified by the antioxidant system. The most widely accepted theory of apoptosis induction postulates that the enhanced generation of ROS induces opening of the mitochondrial megachannels known as permeability transition pores [31, 35, 36, 37]. Disruption

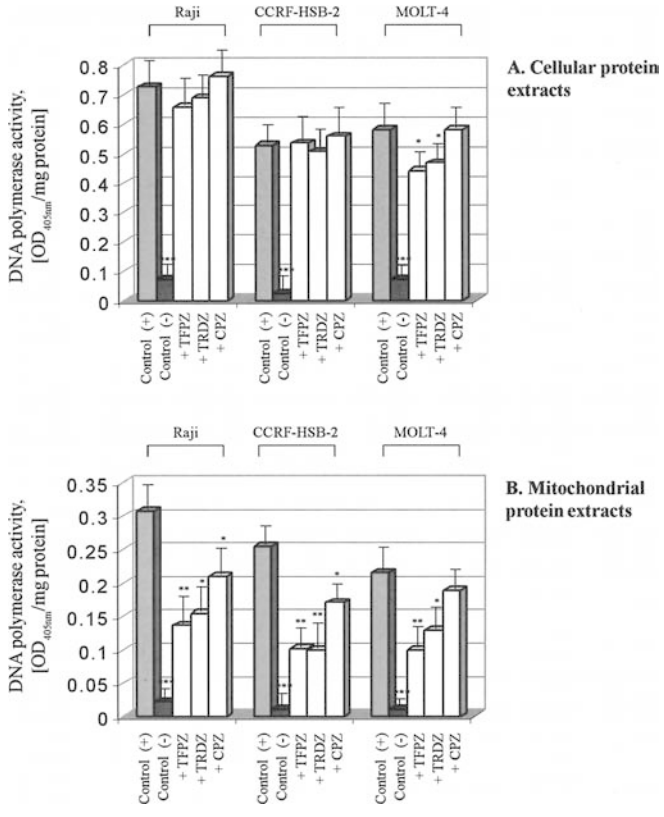

Fig. 5A, B Effect of phenothiazines on DNA polymerase activity in native leukemic cells (**A**) and isolated mitochondria (**B**). Leukemic cells (Raji, CCRF-HSB-2 or MOLT-4) were treated with 10 μM phenothiazine (TFPZ, TRDZ or CPZ) for 24 h. Untreated cells were used as positive controls. Protein extracts from cells or mitochondrial fraction were prepared and aliquots were used for DNA polymerase activity assay as described in Material and methods. Protein extracts, pretreated with 100 m*M* ammonium sulfate, which is known to suppress eukaryotic DNA polymerases, were used as negative controls. The results are presented as the means ± SD from four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, vs the control group

of the mitochondrial membrane potential ($d\psi_m$) takes place, which is accompanied by release of cytochrome *c* from the mitochondria to the cytoplasm [31, 37, 38]. The cytoplasmic cytochrome *c* serves as a cofactor with apoptotic protease activating factor (Apaf-I) to activate the caspase cascade, which undermines the structural integrity of the cells by cleaving key structural protein substrates leading to DNA fragmentation [32, 39]. This "classical" pathway of apoptosis takes place in normal cells as well as in cancer cells.

Our results showed that the phenothiazines (up to 20 μ*M*) induced a decrease in ATP production in leukemic cells and probably influenced mitochondrial function and integrity, but had no significant effect on ATP synthesis in normal lymphocytes. Therefore, phenothiazine-mediated induction of apoptosis in leukemic cells through the "classical" mechanism, described above, is a controversial question. There are at least three arguments in support of this assumption. First, cancer cells are known to show a stronger antioxidant defense system and higher resistance to ROS than normal cells. Second, phenothiazines are well-known antioxidants [32, 40, 41]. Moreover, TRDZ, TFPZ and CPZ, which show a well-expressed antioxidant activity greater than that of PMZ and LVPZ (reported by us previously) [40, 41], also showed considerable cytotoxic and apoptotic activity (Figs. 2 and 3). Third, TFPZ, TRDZ and CPZ did not affect cytochrome *c* release in Raji, CCRF-HSB-2 or MOLT-4 cells during 24 h of incubation (Fig. 4A), but marked DNA fragmentation was detected during this time period (Fig. 3). Moreover, TFPZ and TRDZ suppressed Ca^{2+}-induced cytochrome *c* release from isolated mitochondria (Fig. 4B) and markedly prevented Ca^{2+}-induced mitochondrial swelling (data are not shown). CPZ did not influence significantly either of these processes.

Another hypothetical mechanism for cancer cell selectivity of phenothiazines can be postulated, proceeding from data in the literature. Phenothiazines are known to inhibit Ca^{2+}-calmodulin binding proteins, which have been found to associate with DNA polymerase isoenzymes. The drugs inhibit DNA polymerase activity and suppress DNA synthesis [20, 42]. In mitochondria, these events are crucial for their normal function, which has to guarantee the production of enough energy. Cancer cells are distinguished from normal ones by their high need for ATP and by their higher sensitivity to a decreased energy supply. Therefore, the potential inhibition of mitochondrial DNA polymerase and DNA synthesis by phenothiazines may explain, at least partially, the difference in the effect of these drugs on the viability of normal and leukemic cells observed in our study. Even though this hypothesis is speculative, it seems most likely and needs verification. The same mechanism has already been postulated for the antiviral activity of phenothiazines in pure viral systems [43, 44, 45, 45].

TFPZ, TRDZ and CPZ did not significantly affect DNA polymerase activity in protein extracts obtained from native Raji and CCRF-HSB-2 cells. Poor inhibition was detected in MOLT-4 cells treated with TFPZ or TRDZ. However, the drugs markedly inhibited DNA polymerase activity in protein extracts obtained from mitochondrial fractions of leukemic cells (Fig. 5). These results support our assumption of phenothiazine-dependent induction of apoptosis in leukemic cells through a "nonclassical" pathway, including suppression of mitochondrial homeostasis. Future investigations in this area will provide important information for the elucidation of the mechanism of the anticancer activity of phenothiazines, as well as for the detailed elucidation of their anti-multidrug resistance effect.

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