Thioridazine Induces Apoptosis of Multidrug-resistant Mouse Lymphoma Cells Transfected with the Human *ABCB1* and Inhibits the Expression of P-Glycoprotein

GABRIELLA SPENGLER[1,3], JOSEPH MOLNAR[1], MIGUEL VIVEIROS[2,4] and LEONARD AMARAL[2,3,4]

[1]*Institute of Medical Microbiology and Immunobiology, Faculty of Medicine,*
University of Szeged, Szeged, Hungary;
[2]*Group of Mycobacteriology, Unit of Microbiology and* [3]*Unit of Parasitology and Medical Microbiology (UPMM),*
Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisboa, Portugal;
[4]*Cost Action BM0701 (ATENS) of the European Commission, Brussels, Belgium*

Abstract. *Aim: Chlorpromazine has activity against a large variety of cancer types. However, this phenothiazine produces a plethora of serious side-effects. We have studied thioridazine (TZ), a phenothiazine neuroleptic that is much milder, for activity against multidrug-resistant (MDR) cancer cells, as well as against the overexpressed ABCB1 transporter (P-glycoprotein) that is the cause for the MDR phenotype of these cancer cells. Materials and Methods: MDR mouse T-lymphoma cells, transfected with the human gene ABCB1 that codes for the transporter ABCB1, were incubated with TZ for various periods of time and examined for evidence of apoptosis. Concentrations of TZ were evaluated for activity against the ABCB1 transporter with the aid of an automated ethidium bromide (EB) method. Results: TZ induces apoptosis of MDR cancer cell line, as well as inhibits the activity of the overexpressed ABCB1 transporter of these cells. Conclusion: Because thioridazine has been in moderately safe use for over 40 years for the therapy of psychosis, it has the potential to serve as an adjuvant with anticancer agents, rendering the a priori MDR cancer cells susceptible to the anticancer agent.*

In the developing world, survival rates from cancer are far lower due to poor diagnostic facilities, hence diagnoses are made during late stages of cancer often precluding any form of therapeutic success, poor competence in diagnostic and therapeutic aspects, and low availability of effective but also costly forms of therapy (chemotherapy, radiotherapy). Regardless of economic support, chemotherapy of cancer is highly problematic, especially when therapy promotes the development of multidrug resistance (MDR), hence cancer becomes refractory not only to the initial chemotherapeutic agent, but to many other anticancer drugs as well (1, 2). The development of an MDR phenotype of cancer cell has been known for many decades to be due to the overexpression of transporters that extrude the anticancer agent before it reaches its intended target (3). Consequently, there has been an intensive and extensive search for agents that inhibit the transporter, thereby rendering the cancer susceptible to any anticancer agent. As an example, over 100,000 compounds have been screened by the National Cancer Institute (NCI) for activity against MDR transporters of 60 cancer cell lines (4). However, because these transporters are also present and vital to normal cells, as of the time of this writing, no inhibitor of any transporter responsible for the MDR phenotype of cancer has successfully passed clinical evaluation.

Transporters that render cancer cells multidrug-resistant. When tumor resistance develops against a single particular chemotherapeutic agent, in many cases the resulting phenotype shows a wide range of resistance to that chemotherapeutic agent as well as to many structurally unrelated noxious compounds (MDR pattern) (5). The major mechanism of MDR is the expression of ATP-dependent transporters known as the ATP-binding cassette (ABC) family (6). In humans, the three major types of MDR proteins include members of the ABCB (ABCB1/MDR1/p-glycoprotein), the ABCC (ABCC1/MRP1, ABCC2/MRP2, probably also ABCC3–6, and ABCC10–11), and the ABCG (ABCG2/MXR/BCRP) subfamily. On the basis of a great

Correspondence to: Leonard Amaral, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal. Tel: +351 213652600, Fax: +351 213632105, e-mail: lamaral@ihmt.unl.pt

Table I. *Apoptosis-inducing activity of thioridazine on MDR mouse T-lymphoma cells after 90 min.*

Induction for 90 min	Concentration μg/ml	Gated events (%)		
		Early apoptosis	Late apoptosis and necrosis	Cell death
Control A– I–		0	0	0
Control A–I+		0	0	0.39
Control A+I–		0.68	0	0
Control A+I+		0.50	0.14	0.67
Control DMSO	1%	1.50	0.32	0.82
12H-Benzo[α]phenothiazine (M627)	50	24.76	25.34	1.72
Thioridazine	5[a]	15.49	6.64	0.67
	10[b]	49.54	27.26	3.40
	20[c]	Toxicity (100% dead cells)		

Control A–I–, Control A–I+, Control A+I– and Control A+I+ are internal controls of the Annexin V-FITC Apoptosis Detection Kit. DMSO is used routinely as control since other agents assayed by the system and not included in these results have low solubility in the medium. These controls indicate that the culture system and period of incubation do not produce any significant apoptosis, necrosis or cell death as per the limits expressed by the kit. [a]Represents the lowest concentration of agent that produced significant apoptosis, necrosis and little cell death; [b]represents the highest concentration of agent that produced significant apoptosis, necrosis and because a significant percentage of cells are dead, this concentration produces evidence of a low level of toxicity; [c]produces clear toxicity, 100% of the cell population is dead.

deal of clinical and experimental work, it has been established that these pumps recognize a very wide range of unrelated drug substrates. ABCB1 preferentially extrudes large hydrophobic molecules, while ABCC1 and ABCG2 can transport both hydrophobic drugs and large anionic compounds, *e.g.* drug conjugates (7). The investigation of the relationship between ABC expression levels and sensitivity to drugs or possible drug candidates is of great importance because an agent that is eventually shown to inhibit an ABC transporter of a cancer cell line can contribute to further overexpression of other ABC transporters responsible for the MDR phenotype (8).

The activity of phenothiazines towards cancer cells. Phenothiazines have been shown to have neuroleptic, antimycobacterial and anticancer properties (19-17). It has been demonstrated that chlorpromazine (CPZ) inhibits the growth of cancer cells whenever studied (17-21). Moreover, other phenothiazines such as promethazine (22) and thioridazine (23-25) also inhibit a variety of cancer types. In fact, the properties of phenothiazines associated with inhibitory effects on cancer cell growth can be reproduced with inhibitors of calmodulin (26-29). These studies suggest that calcium signaling pathways and calcium-dependent enzymes are targets of phenothiazines (30). Because some of these pathways are involved in the generation of ATP, and ATP is required for the activity of ABC transporters which, when overexpressed, result in the MDR phenotype of cancer cells, it is obvious that studies that focus on the activity of phenothiazines towards the ABC transporters of cancer cells are needed. Since unlike CPZ, thioridazine (TZ) is much milder and produces fewer negative side-effects than

CPZ (30), we have studied the activity of this phenothiazine on the viability of MDR mouse lymphoma cells transfected with the human *ABCB1* gene that codes for ABCB1, the transporter that when overexpressed, renders the cell immune to anticancer agents (31-33), as well as on the activity of the ABCB1 transporter itself.

Materials and Methods

Cell lines. L5178 mouse T-cell lymphoma cells (ECACC cat. no. 87111908, U.S. FDA, Silver Spring, MD, USA) were transfected with pHa ABCB1/A retrovirus (31-33), as described elsewhere (34, 35). The *ABCB1*-expressing cell lines were selected by culturing the infected cells with 60 ng/ml of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maintain the MDR phenotype. L5178 mouse T-cell lymphoma cells (parental) and the human *ABCB1*-gene transfected sub-line were cultured in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich), L-glutamine (Sigma-Aldrich) and antibiotics (penicillin, streptomycin) (Sigma-Aldrich) at 37°C and in an atmosphere with 5% CO_2.

Compounds. Ethidium bromide (EB), verapamil, reserpine and TZ were purchased from Sigma-Aldrich Quimica SA, Madrid, Spain.

Viability assay. The cytotoxicity of EB and the efflux pump inhibitors (EPIs), verapamil, reserpine and TZ was determined by the trypan blue exclusion method, previously described in detail (36). The effect of the aforementioned agents on apoptosis was determined with the aid of the Annexin V-FITC Apoptosis Detection Kit (Calbiochem EMD Biosciences, Inc. La Jolla, CA, USA) strictly in accordance with manufacturer's instructions (31-33).

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
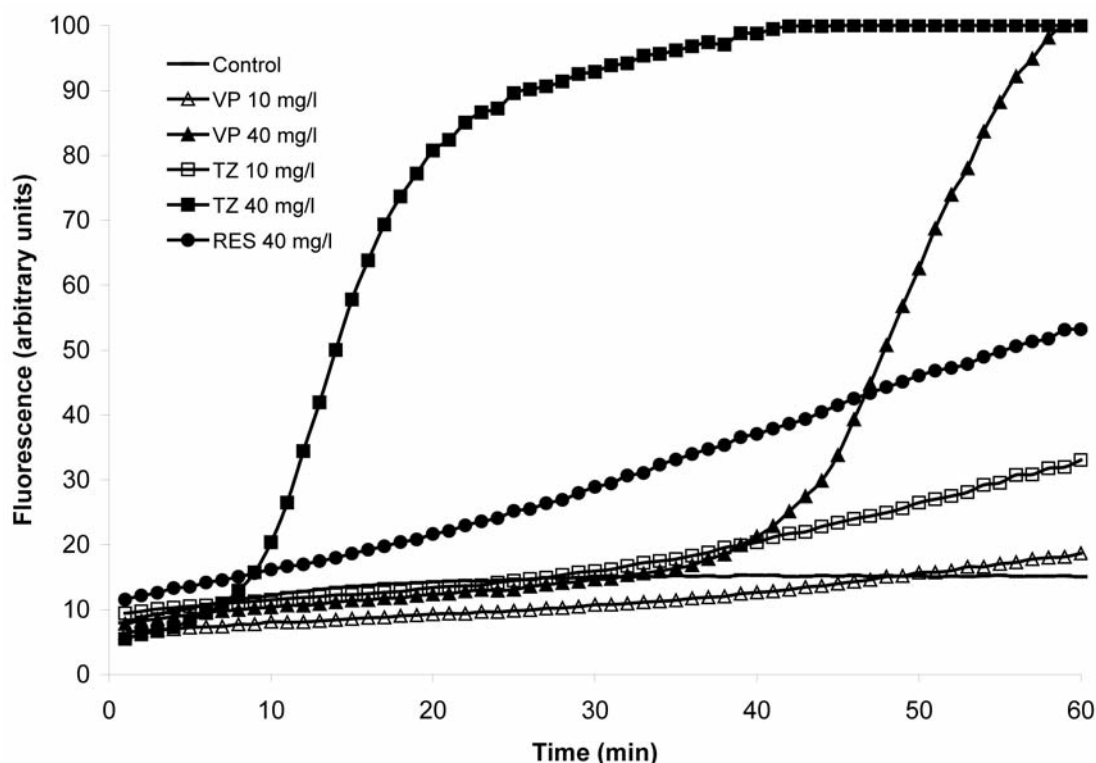
Figure 1. *Ethidium bromide accumulation by MDR mouse T-lymphoma cells overexpressing the ABCB1 transporter in the presence of verapamil (VP), thioridazine (TZ) and reserpine (RES). Cells (2×10^6 cells/ml) were suspended in 100 μl of phosphate-buffered saline solutions (pH 7.4) supplemented with 1 mg/l of EB with and without agent at given concentrations, then the fluorescence was continuously monitored.*

phosphate-buffered saline (PBS) solutions of pH 7.4 supplemented with glucose. The cell suspension was distributed in 90 μl aliquots into 0.2 ml tubes. The tested compounds were individually added at different concentrations in 5 μl volumes of their stock solutions and the samples were incubated for 10 min at 25°C. 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 into a Rotor-Gene™ 3000 thermocycler (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), the 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. The results were evaluated by Rotor-Gene Analysis Software 6.1 (Build 93) provided by Corbett Research (Sydney, Australia). A complete description of the method has been previously presented in detail (31-33). The essence of the method is that a progressive increase of fluorescence, induced by the agent in question, provides an estimate of the inhibition of efflux of EB promoted by that agent.

Assay for apoptosis induction. The assay was carried out using Annexin V-FITC Apoptosis Detection Kit from Calbiochem (EMD Biosciences, Inc. La Jolla, CA, USA) according to the manufacturer's instructions. The cell suspension was adjusted to approximately 1×10^6 cells/ml. The cell suspension was distributed into 0.5 ml aliquots (5×10^5 cells) and transferred to Eppendorf tubes. The cells were incubated in the presence of the compounds for 90 min at 37°C, with

$12H$-benzo[α]phenothiazine (M627) being used as positive control at a final concentration of 50 μg/ml (37), then the samples were washed in PBS. The cells were centrifuged at 2000×g for 3 minutes. The harvested cells were re-suspended in culture medium and distributed to 24-well tissue culture plate in 0.5 ml aliquots, followed by the incubation of the plate for 24 h at 37°C in 5% CO_2. After the incubation, the apoptosis assay was carried out according to the rapid protocol of the kit. The fluorescence was analyzed immediately using a Becton Dickinson FACStar™ flow cytometer (Becton Dickinson and Company, USA).

Results and Discussion

The induction of apoptosis of the MDR mouse lymphoma cells by concentrations of TZ is presented in Table I. Briefly, concentrations below the ones that, produce toxicity (20 mg/l) induced apoptosis in a concentration-dependent manner.

To assess the effect of TZ on apoptosis of MDR mouse T-lymphoma cells, after determination of the cytotoxic activity of thioridazine (IC50 9.56 μg/ml after 24 h), three concentrations of TZ (5, 10 and 20 μg/ml) were selected for apoptosis studies. During apoptosis, the plasma membrane undergoes multiple changes: the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane can be detected by annexin V. To

discriminate between apoptotic and necrotic cells, the annexin V-FITC binding assay was combined with propidium iodide staining, but due to increased membrane permeability, late apoptotic cells cannot be discriminated from necrotic cells. Apoptosis was measured by quantifying the proportion of annexin V-FITC and propidium iodide positive cells using flow cytometry. As shown in Table I, the treatment with 5 μg/ml TZ for 90 min induced early apoptosis in 15.49% of the cell population and 6.6% of the cells underwent late apoptosis. The apoptosis-inducing potential of TZ was more potent at the concentration of 10 μg/ml, because 49.54% of the cells were early-apoptotic and 27.26% showed late-apoptotic and necrotic features, although the proportion of dead cells was 3.4%. The induction with 20 μg/ml of TZ was toxic for the population.

The evaluation of agents and TZ for activity against the overexpressed ABCB1 transporter of the MDR mouse lymphoma cells is depicted in Figure I. Briefly, verapamil and reserpine that serve as positive controls inhibit ABCB1 activity in a concentration-dependent manner. In comparison to these effects, TZ inhibits the activity of ABCB1 in a similar concentration-dependent manner and on a μg/ml basis, the inhibitory activity is far greater than that of verapamil and reserpine.

The results obtained in the current study suggest that TZ induces apoptosis of the MDR lymphoma cells and inhibits the activity of their overexpressed ABCB1 transporter. However, the concentrations that produce these results are extremely high and greater than the ones used for the therapy of psychosis – namely, *ca*. 0.5 μg/ml. Nevertheless, TZ can serve as a lead compound for the development of derivatives which may be more active at concentrations that are non-toxic and achievable in the patient. At the time of this writing, these derivatives have been produced and will be the subject of further studies.

Acknowledgements

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61(2)*: 69-90, 2011.

2 Amaral L, Engi H, Viveiros M and Molnar J: Comparison of multidrug-resistant efflux pumps of cancer and bacterial cells with respect to the same inhibitory agents. In Vivo *21*: 237-244, 2007.

3 Moitra K and Dean M: Evolution of ABC transporters by gene duplication and their role in human disease. Biol Chem *392*: 29-37, 2011.

4 Wu CP, Calcagno AM and Ambudkar SV: Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. Curr Mol Pharmacol *1*: 93-105, 2008

5 Ferguson LR and De Flora S: Multiple drug resistance, antimutagenesis and anticarcinogenesis. Mutat Res *591*: 24-33, 2005.

6 Higgins CF: ABC transporters: from microorganisms to man. Annu Rev Cell Biol *8*: 67-113, 1992.

7 Sarkadi B, Homolya L, Szakács G and Váradi A: Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol Rev *86*: 1179-1236, 2006.

8 Szakács G, Annereau JP, Lababidi S, Shankavaram U, Arciello A, Bussey KJ, Reinhold W, Guo Y, Kruh GD, Reimers M, Weinstein JN and Gottesman MM: Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. Cancer Cell *6*: 129-137, 2004.

9 Gérard Taurand: Phenothiazine and Derivatives. *In*: Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH, Weinheim, 2005.

10 Ehrlich P: Papers reprinted. *In*: The Collected Papers of Paul Ehrlich. Himmelweit F, Marquardt M and Dale HH (eds.), Vol III. Chemotherapy: London: Pergamon pp. 505-518, 1913.

11 Healy D: The Creation of Pharmacology. Harvard University Press, Boston, MA, 2002.

12 Kristiansen JE and Amaral L: The potential management of resistant infections with non-antibiotics. J Antimicrob Chemother *40*: 319-327, 1997.

13 Amaral L and Kristiansen JE: Phenothiazines: an alternative to conventional therapy for the initial management of suspected multidrug resistant tuberculosis. A call for studies. Int J Antimicrob Agents *14*: 173-176, 2000.

14 Amaral L, Kristiansen JE, Viveiros M and Atouguia J: Activity of phenothiazines against antibiotic-resistant *Mycobacterium tuberculosis*: a review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. J Antimicrob Chemother *47*: 505-511, 2001.

15 Amaral A, Kristiansen JE: Phenothiazines: potential management of Creutzfeldt-Jacob disease and its variants. Int J Antimicrob Agents *18*: 411-417, 2001.

16 Amaral L, Viveiros M and Kristiansen JE: Phenothiazines: potential alternatives for the management of antibiotic resistant infections of tuberculosis and malaria in developing countries. Trop Med Int Health *6*: 1016-1022, 2001.

17 Darkin S, McQuillan J and Ralph RK: Chlorpromazine: a potential anticancer agent? Biochem Biophys Res Commun *125*: 184-191, 1984.

18 Akiyama S, Shiraishi N, Kuratomi Y, Nakagawa M and Kuwano M: Circumvention of multiple-drug resistance in human cancer cells by thioridazine, trifluoperazine, and chlorpromazine. J Natl Cancer Inst *76*: 839-844, 1986.

19 Sagar PS, Das UN, Koratkar R, Ramesh G, Padma M and Kumar GS: Cytotoxic action of cis-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. Cancer Lett *63*: 189-198, 1992.

20 Aas AT, Brun A, Pero RW and Salford LG: Chlorpromazine in combination with nitrosourea inhibits experimental glioma growth. Br J Neurosurg *8*: 187-192, 1994.

21 Hait WN, Gesmonde JF and Lazo JS: Effect of anti-calmodulin drugs on the growth and sensitivity of C6 rat glioma cells to bleomycin. Anticancer Res *14*: 1711-1721, 1994.

22 Jones GR: Successful cancer therapy with promethazine: the rationale. Med Hypotheses 46: 25-29, 1996.

23 Rho SB, Kim BR and Kang S: A gene signature-based approach identifies thioridazine as an inhibitor of phosphatidylinositol-3'-kinase (PI3K)/AKT pathway in ovarian cancer cells. Gynecol Oncol *120*: 121-127, 2011.

24 Gil-Ad I, Shtaif B, Levkovitz Y, Nordenberg J, Taler M, Korov I and Weizman A: Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate in vivo melanoma tumor growth. Oncol Rep *15*: 107-112, 2006.

25 Strobl JS and Peterson VA: Tamoxifen-resistant human breast cancer cell growth: inhibition by thioridazine, pimozide and the calmodulin antagonist, W-13. J Pharmacol Exp Ther *263*: 186-193, 1992.

26 Mayur YC, Peters GJ, Prasad VV, Lemo C and Sathish NK: Design of new drug molecules to be used in reversing multidrug resistance in cancer cells. Curr Cancer Drug Targets *9*: 298-306, 2009.

27 Colomer J, Means AR: Physiological roles of the Ca^{2+}/CaM-dependent protein kinase cascade in health and disease. Subcell Biochem *45*: 169-214, 2007.

28 Mayur YC, Jagadeesh S and Thimmaiah KN: Targeting calmodulin in reversing multi-drug resistance in cancer cells. Mini Rev Med Chem *6*: 1383-1389, 2006.

29 Rodriguez-Mora O, LaHair MM, Howe CJ, McCubrey JA and Franklin RA: Calcium/calmodulin-dependent protein kinases as potential targets in cancer therapy. Expert Opin Ther Targets *9*: 791-808, 2005.

30 Amaral L, Kristiansen JE, Abebe LS and Millett W: Inhibition of the respiration of multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* by thioridazine: potential use for initial therapy of freshly diagnosed tuberculosis. J Antimicrob Chemother *38*: 1049-1053, 1996.

31 Spengler G, Evaristo M, Handzlik J, Serly J, Molnár J, Viveiros M, Kiéc-Kononowicz K and Amaral L: Biological activity of hydantoin derivatives on P-glycoprotein (ABCB1) of mouse lymphoma cells. Anticancer Res *30*: 4867-4871, 2010.

32 Spengler G, Ramalhete C, Martins M, Martins A, Serly J, Viveiros M, Molnár J, Duarte N, Mulhovo S, Ferreira MJ and Amaral L: Evaluation of cucurbitane-type triterpenoids from Momordica balsamina on P-glycoprotein (ABCB1) by flow cytometry and real-time fluorometry. Anticancer Res *29*: 3989-3993, 2009.

33 Spengler G, Viveiros M, Martins M, Rodrigues L, Martins A, Molnar J, Couto I and Amaral L: Demonstration of the activity of P-glycoprotein by a semi-automated fluorometric method. Anticancer Res *29*: 2173-2177, 2009.

34 Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC: A retrovirus carrying an *MDR1* cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Natl Acad Sci USA *85*: 4486-4490, 1988.

35 Choi K, Frommel TO, Stern RK, Perez CF, Kriegler M, Tsuruo T and Roninson IB: Multidrug resistance after retroviral transfer of the human *MDR1* gene correlates with P-glycoprotein density in the plasma membrane and is not affected by cytotoxic selection. Proc Natl Acad Sci USA *88*: 7386-7390, 1991.

36 Ordway D, Hohmann J, Viveiros M, Viveiros A, Molnar J, Leandro C, Arroz MJ, Gracio MA and Amaral L: *Carpobrotus edulis* methanol extract inhibits the MDR efflux pumps, enhances killing of phagocytosed *S. aureus* and promotes immune modulation. Phytother Res *17*: 512-519, 2003.

37 Mucsi I, Varga A, Kawase M, Motohashi N and Molnar J: Interaction between various resistance modifiers and apoptosis inducer 12*H*-benzo[alpha]phenothiazine. Anticancer Res *22(5)*: 2833-2836, 2002.