

Inhibition of O⁶-Alkylguanine DNA-Alkyltransferase or Poly(ADP-ribose) Polymerase Increases Susceptibility of Leukemic Cells to Apoptosis Induced by Temozolomide

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SUMMARY

High levels of expression of the DNA repair enzyme O⁶-alkylguanine DNA-alkyltransferase (OGAT) (EC 2.1.1.63) account for tumor cell resistance to methylating agents. Previous studies suggested that methylating triazenes might have a potential role for the treatment of acute leukemias with low levels of OGAT. In the current study, we transduced the human OGAT cDNA in OGAT-deficient leukemia cell clones. OGAT-transduced cells were more resistant than their OGAT-deficient counterparts to apoptosis triggered by the methylating triazene temozolomide (TZM), as indicated by the results of flow cytometry, terminal deoxynucleotidyl transferase assay, and analysis of DNA fragmentation. Depletion of OGAT activity by O⁶-benzylguanine increased leukemia cell sensitivity to TZM-mediated

apoptosis. Moreover, combined treatment of cells with TZM and benzamide, an inhibitor of the poly(ADP-ribose) polymerase (EC 2.4.2.30), increased the apoptosis induced by the methylating agent. These results demonstrate for the first time that methyl adducts at the O⁶ position of guanine, which are specifically removed by OGAT, are the principal DNA lesions responsible for the induction of apoptosis on treatment of leukemic cells with the methylating triazene TZM. This study also supports the possible use of TZM for the treatment of acute leukemias and suggests new strategies to increase the susceptibility of tumor cells to methylating triazenes in the clinic.

Tumor cell resistance to alkylating agents is generally mediated by DNA repair enzymes, including OGAT; excision repair (1); and mismatch repair systems (2) and by high levels of glutathione and glutathione-S-transferase (3) or polyamines (4). Among these factors, the direct repair of DNA adducts by OGAT represents the principal and best studied mechanism of cellular protection from DNA damage by methylating and chloroethylating agents (5, 6). This enzyme transfers the O⁶-alkylguanine adducts to a cysteine residue in a stoichiometric and autoinactivating reaction (7).

Recently, OGAT was found to play an important role in resistance of human leukemic cells to the tetrazine TZM (8). Moreover, a previous clinical study showed that the triazene compound dacarbazine induces a marked reduction of leukemic blasts in patients with refractory or relapsed acute myelogenous leukemias characterized by low OGAT levels, thus

suggesting a potential role of this drug for leukemia treatment (9).

The main problem concerning dacarbazine is its cumulative bone marrow toxicity, which seems to be, at least in part, overcome by the introduction into clinical trials of TZM, which shows limited hematological toxicity. This agent does not require metabolic activation because it spontaneously decomposes in aqueous solution to give the reactive methylating species 5-(3-methyl-1-triazeno)imidazole-4-carboxamide, which is the active metabolite of dacarbazine (10).

Methylating agents interact with DNA and generate methyl adducts at several DNA sites. Their mutagenic and cytotoxic properties are mainly due to their ability to methylate the O⁶ position of guanine (11). Unrepaired O⁶-methylguanine is a mutagenic lesion responsible for G:C-to-A:T transition. This generates a point mutation that is involved in the appearance of novel immunogenic peptide(s) (i.e., chemical xenogenization), as shown in murine leukemic cells

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ABBREVIATIONS: OGAT, O⁶-alkylguanine DNA-alkyltransferase; TZM, temozolomide; PADPRP, poly(ADP-ribose) polymerase; BG, O⁶-benzylguanine; BZ, benzamide; CM, complete medium; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; b-dUTP, biotin-16-dUTP; PFGE, pulsed field gel electrophoresis; kb, kilobase pair(s).

treated with methyl-triazenes (12). Moreover, it is possible that a point mutation located in genes essential for cell growth and/or survival would be lethal for the cells. However, the main mechanism underlying the cytotoxic effects generated by DNA O⁶-methylguanine relies on the induction of aberrant mismatch repair processes, which generate DNA strand breaks (13).

The enzyme PADPRP is activated by DNA strand breaks (for a review, see Ref. 14). Its function has been implicated in a variety of biological processes, including DNA repair and cell survival after DNA damage. Interaction of PADPRP with DNA might allow access of the repair enzymes to the damaged sites on DNA. It has been shown that PADPRP inhibitors have the potential to act as resistance modifiers when used in combination with chemotherapeutic agents (15).

Proteolytic cleavage of PADPRP has been considered an early marker of apoptosis (16). The cleavage prevents the catalytic domain of the enzyme from coordinating DNA repair. In addition, PADPRP-mediated ribosylation seems to regulate negatively the Ca²⁺/Mg²⁺-dependent endonuclease involved in apoptosis (for a review, see Ref. 16).

Recently, we have shown that apoptosis is involved in TZM-induced cytotoxicity against human leukemic lines (17). In the current study, the molecular mechanisms possibly involved in leukemic cell resistance to TZM were investigated. K562 and U937 OGAT-deficient leukemia cell clones were transfected with the human OGAT cDNA by using an amphotropic virus. This model has been used to explore different strategies to increase leukemic cell sensitivity to apoptosis induced by TZM. The results pointed out that (a) apoptosis induced by TZM is prevented by target cell transfection with OGAT; (b) depletion of OGAT by BG (18), a specific inhibitor of OGAT enzyme, potentiates the cytotoxic and apoptotic effects induced by TZM in OGAT-transduced leukemic cells; and (c) combined treatment with TZM and BZ, an inhibitor of PADPRP, results in overall increase in leukemic cell apoptosis in OGAT-proficient or -deficient cells.

Materials and Methods

Cell lines. Clones were obtained by limiting dilution of the human leukemia cell lines K562, an erythroleukemia cell line (American Type Culture Collection, Rockville, MD) and U937, a histiocytic cell line (American Type Culture Collection). These cell lines were cultured at 37° in 5% CO₂ humidified atmosphere and maintained in CM [RPMI-1640 (GIBCO, Paisley, Scotland, UK) supplemented with 10% heat-inactivated (56°, 30 min) fetal calf serum (GIBCO), 2 mM L-glutamine, and antibiotics (Flow Laboratories, McLean, VA)].

The amphotropic packaging cell line PA317 and NIH 3T3 cells were obtained from Dr. J. Schlom (National Cancer Institute, National Institutes of Health, Bethesda, MD). PA317 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum and L-glutamine.

Transfection and transduction of OGAT cDNA. A *Hind*III fragment, encompassing the human OGAT cDNA (19), was cloned into the retroviral vector pLNSX (20). After DNA sequencing by the chain termination method using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH), these constructs or the vector pLNSX was transfected into the PA317 packaging cell line according to the calcium phosphate precipitation procedure. Stable virus producing cell lines were generated after selection of the mass culture with 1 mg/ml G418. The apparent virus titers were determined on murine NIH 3T3 cells by G418 selection (27) and ranged between 10⁻⁴ and 4 × 10⁻⁴ colony-forming units/ml. U937 or K562 clones, in

the exponential phase of growth, were transduced by replacing culture medium with a 1:1 mixture of OGAT recombinant or control virus producing PA317 cells and fresh CM with the addition of polybrene (8 µg/ml). Two days after transduction, cells were G418 selected (1 mg/ml). The nontransduced control cells died at this antibiotic concentration.

Reagents. TZM was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). ³H-methylated DNA, to be used as substrate for OGAT activity, was prepared using [³H]methyl-N-nitrosourea (specific activity, 19.2 Ci/mmol; Amersham International, Amersham, UK) (21). BG was synthesized and kindly given by Dr. L. Lassiani (Institute of Pharmacological Chemistry, University of Trieste, Trieste, Italy). Etoposide (VP16) and BZ were purchased from Bristol-Myers Squibb (Rome, Italy) and Sigma (St. Louis, MO), respectively. TZM and BZ stock solutions were prepared by dissolving the drugs in RPMI-1640, whereas BG was dissolved in ethanol.

RT-PCR. Total RNA was isolated by extraction according to the guanidinium thiocyanate method and treated with DNase to eliminate the possibility of genomic DNA contamination. cDNA was synthesized by incubating 2 µg of RNA with 25 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) at 37° for 1 hr in the presence of 50 pmol of oligo(dT)15. After heat inactivation at 95° for 5 min of the enzyme, aliquots of the synthesized first-strand cDNA, for each sample, were used as template for PCR amplification. Human OGAT or GAPDH amplification was performed in DNA thermal cycler for 30 cycles (Perkin-Elmer Cetus, Norwalk, CT; denaturation at 95° for 1 min, annealing at 60° for 1 min, and extension at 72° for 2 min, for each cycle). The oligonucleotide primers pairs used for OGAT amplification were 5'-GAGGCCTAGGCTTTGCAA-3' and 5'-TATAAGCTTACTCAGTTTCGCCAGCAGG-3', which were complementary to the pLNSX vector sequences upstream of the *Hind*III cloning site and to the 3' end of the transcribed strand of the human OGAT coding sequences (22). These primers allow amplification of only the DNA complementary to the virus-derived OGAT transcript.

Drug treatment and cell growth evaluation. Cell suspensions containing 1 × 10⁶ cells/ml in CM were placed in 50-ml tubes (Falcon, Becton Dickinson Labware, Oxnard, CA), and TZM was added to each tube at final concentrations of 6.2–200 µM. Cells were then incubated at 37° for 4 hr in the dark. At the end of the incubation period, cells were washed twice with RPMI-1640 and cultured in flasks (Falcon) at a concentration of 1 × 10⁵/ml at 37° in a 5% CO₂ humidified atmosphere. Cell growth was evaluated in terms of viable cell count every 24 hr for 3 days. Cells were manually counted using a hemocytometer, and cell viability was determined by trypan blue exclusion or flow cytometric analysis of cells stained with PI. All determinations were made in quadruplicate. Cell line chemosensitivity to TZM was evaluated in terms of IC₅₀ (i.e., concentration of the drug expressed in µM capable of inhibiting cell growth by 50%). The IC₅₀ values were calculated on the regression line in which the number of viable cells was plotted versus the logarithm of drug concentration. Depletion of OGAT activity was obtained by treating tumor cells (1 × 10⁶/ml in CM) with 1 µM BG for 2 hr before TZM exposure. BG was then added again every 24 hr. Inhibition of PADPRP was obtained by treating tumor cells with BZ (5 or 10 mM) (15).

Assay for OGAT activity. This assay was performed as described by Morten and Margison (23). Briefly, leukemic cells were resuspended at the concentration of 4 × 10⁶ cells/ml in assay buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 3 mM dithiothreitol) and then sonicated (power output, 20 W) in a Vibra Cell VC 600 Sonicator (Sonics and Material, Danbury, CT) using two 15-sec pulses with a 30-sec cooling phase between each pulse. After sonication, phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM. The sonicates were then microfuged at 15,000 rpm at 4° for 10 min to pellet cell debris, and supernatants were used for the assay. OGAT activity was determined by measuring the transfer of ³H-labeled methyl groups from calf thymus DNA that had been previously

methyated by reaction with *N*-[³H]methyl-*N*-nitrosourea. OGAT activity was expressed in femtomoles per milligram of protein.

Assessment of apoptosis by flow cytometry analysis. Cells from cultures were washed with PBS and fixed in 70% ethanol at -20° for 18 hr. The centrifuged pellets were resuspended in 1 ml of hypotonic solution containing 50 µg/ml PI, 0.1% sodium citrate, 0.1% Triton-X, and 10 µg/ml RNase. Cells were incubated in the dark at room temperature for 30 min. Data collection was gated using forward light scatter and side light scatter to exclude cell debris and cell aggregates. The PI fluorescence was measured on a linear scale using a FACSScan flow cytometer (Becton Dickinson, San Jose, CA). Apoptotic cells are represented by a broad hypodiploid peak, which is easily discriminated from the narrow peak of cells with diploid DNA content in the red fluorescence channel (17). All data were recorded and analyzed using Lysis II software (Becton Dickinson).

TdT assay. TdT assay was performed as described by Gorczyca (24). Cells (2 × 10⁶) from cultures were washed with PBS and fixed in formaldehyde (1%) in PBS, pH 7.4, on ice for 15 min. After washing in PBS, cells were resuspended in ice-cold 70% ethanol and stored at -20° for 1–3 days. Cells were then washed in PBS and resuspended in 50 µl of a solution containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl, 2.5 mM cobalt chloride, 0.25 mg/ml bovine serum albumin, pH 6.6, 5 units of TdT, and 0.5 nM b-dUTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The cells were incubated in this solution at 37° for 30 min and then rinsed in PBS and resuspended in 100 µl of the staining buffer, which contained 4× standard saline citrate buffer (600 mM NaCl, 60 mM sodium citrate), 0.1% v/v Triton X-100, 5% (w/v) non-fat dry milk, and 10 µg/ml fluorescein isothiocyanate-conjugated avidin (Boehringer Mannheim). Cells were washed twice in PBS and resuspended in 1 ml of a solution containing 50 µg/ml PI, 0.1% sodium citrate, 0.1% Triton-X, and 10 µg/ml RNase and incubated at room temperature in the dark for 30 min before analysis with the FACSScan flow cytometer (24). Data were acquired and analyzed using Lysis II software. The results were displayed as a representative two-dimensional frequency-contour plot of red-versus-green fluorescence. The percentage of cells exhibiting high green fluorescence was determined where the green gate was positioned after examination of control samples.

Analysis of DNA fragmentation by conventional gel electrophoresis. DNA fragmentation assay was performed as previously described (17). Control and drug-treated cells were recovered from cultures after 72 hr, collected by centrifugation, washed twice in PBS, and resuspended (5 × 10⁶) in 0.2 ml of a solution containing 10 mM Tris-HCl, pH 8.6, 1.5 mM MgCl₂, 140 mM NaCl, and 0.5% Nonidet P-40. Samples were incubated for 5 min in ice and subsequently microfuged at 14,000 rpm for 5 min. Supernatants were removed and incubated in the presence of 0.25 mg/ml DNase-free RNase A at 37° for 1 hr. After the addition of 0.2 ml of 2× proteinase K buffer (0.2 M Tris-HCl, pH 8.0, 25 mM EDTA, 0.3 M NaCl, 2% sodium dodecyl sulfate), samples were incubated for 30 sec at 37° in the presence of proteinase K (50 µg/ml). Low-molecular-weight DNA was then extracted once with phenol [buffered with 0.1 M Tris-HCl, pH 7.4/ chloroform/isoamyl alcohol (24:24:1)] and precipitated for 24 hr in the presence of 0.1 M Na acetate, pH 5.2, with 1 volume of isopropanol. The DNA precipitates were recovered by centrifugation at 14,000 rpm using a refrigerated microfuge and analyzed by electrophoresis in 2% agarose gel containing ethidium bromide (0.5 µg/ml). DNA was visualized under UV light and photographed using a Polaroid camera setup.

PFGE. PFGE of high-molecular-weight DNA fragments and preparation of plugs were performed according to Walker (25). Briefly, cells (2 × 10⁶) were harvested from culture; washed twice with ice-cold PBS; resuspended in 1.0 ml of a buffer containing 0.15 M NaCl, 2 mM KH₂PO₄/KOH, pH 6.4, 1 mM EGTA, and 5 mM MgCl₂; centrifuged at 10,000 rpm for 2 min; and washed again twice with 100 µl of the same buffer. Cells were resuspended in 50 µl of this buffer and transferred to an Eppendorf tube containing an equal volume of molten 1.5% low-melting point agarose and 0.4 mg/ml

proteinase K. The mixture was then pipetted in a 1-ml syringe and refrigerated at 4° for 15 min. The agarose blocks were placed in 1 ml of 10 mM NaCl, 10 mM Tris-HCl, pH 9.5, 25 mM EDTA, and 1% *N*-lauroylsarcosine supplemented with 0.1 mg/ml proteinase K and incubated at 37° for 3 hr. Plugs were then rinsed in three changes of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA solution (45 mM Tris/borate and 1 mM EDTA, pH 8.3) at 4° for 10 min each. The agarose blocks were stored in 50 mM EDTA, pH 8.0, for ≤1 week and then used for electrophoresis.

Electrophoresis was carried out using a switch-back power supply (Hoefler Scientific, San Francisco, CA). The gels (1.5% agarose) were run at 200 V at 4° in 0.5× Tris/borate/EDTA containing ethidium bromide (0.5 µg/ml), with the ramping rate changing from T₁ = 0.5 sec and T₂ = 10 sec for 18 hr, and a forward-to-backward ratio of 3. DNA was visualized and photographed as described above for conventional agarose gel electrophoresis.

Results

OGAT transduction decreases cytotoxicity induced by TZM in K562 or U937 clones. Amphotropic PA317 cells were transfected with the construct pLNSX/OGAT or the vector. Supernatants from virus producer PA317 cells were used to infect clonal populations obtained from U937 and K562 cells, both of which are OGAT-deficient cell lines (17). To determine OGAT expression in transfected clones, G418 selected cells were analyzed for the presence of virus-derived OGAT transcript by RT-PCR, as described in Materials and Methods. Fig. 1 shows the results of RT-PCR analysis of representative K562 and U937 clones transfected with pLNSX vector (OGAT-) or the pLNSX/OGAT construct (OGAT+). The presence of the OGAT transcript in K562 or U937 cells transduced with the human OGAT cDNA (Fig. 1, right, lanes 2 and 4) was associated with the presence of OGAT activity that was 10- and 30-fold higher, respectively, than that detected in pLNSX-transduced clones (Table 1). Amplification of the GAPDH cDNA was obtained in all samples (Fig. 1, left).

To establish whether OGAT expression increased resistance to TZM, control or OGAT-transduced cells were exposed to graded concentrations of the drug. The TZM IC₅₀ values, calculated at 72 hr from cell exposure to the drug, indicated that OGAT-expressing K562 or U937 cells were

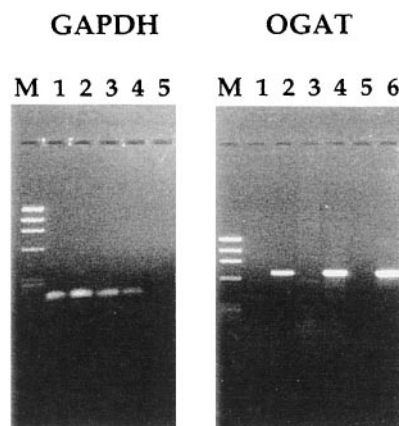


Fig. 1. Analysis of the OGAT transcript in OGAT-transduced leukemia cell clones. RT-PCR for detection of GAPDH (left) or OGAT (right) transcripts was performed with total RNA (2 µg) as described in Materials and Methods. Lane 1, K562/OGAT-. Lane 2, K562/OGAT+. Lane 3, U937/OGAT-. Lane 4, U937/OGAT+. Lane 5, no DNA. Lane 6, pLNSX/OGAT plasmid. M, *Hae* digests of ϕ X174 RF DNA.

TABLE 1
OGAT activity and chemosensitivity to TZM of leukemic cell clones

Values represent the mean \pm standard error of three independent determinations. Cells were exposed to graded concentrations of TZM as described in Materials and Methods. After 72 hr, cells were counted, and IC₅₀ values were calculated on the regression line where the number of cells was plotted versus the log of drug concentration. Probability values were calculated on the basis of Student's *t* test analyses.

	OGAT activity	TZM IC ₅₀	<i>p</i>
	fmol/mg of protein	μ M	
K562 OGAT–	5 \pm 2	15 \pm 3	<0.01
K562 OGAT+	50 \pm 5	69 \pm 5	
U937 OGAT–	9 \pm 2	10 \pm 2	
U937 OGAT+	277 \pm 4	100 \pm 3	<0.01

more resistant to the cytotoxic effects of the agent compared with G418 selected pLNSX-transduced cells (Table 1).

Apoptosis assessment by flow cytometry analysis. To investigate apoptosis involvement in the cytotoxic effects of TZM, untreated or drug-treated OGAT– and OGAT+ cell clones from K562 and U937 were analyzed using flow cytometry, which allowed us to examine changes not only in cell size but also in the fluorescence intensity of the DNA. Cell shrinkage and decrease in fluorescence are both characteristics of apoptosis. In OGAT– clones, after 72 hr of exposure to 12.5 μ M TZM, a fraction of cells became smaller than the controls and showed a simultaneous decrease in DNA content (Table 2). The percentage of OGAT– cells with reduced size and DNA content increased progressively with higher concentrations of the agent (Table 2). The size and DNA content of OGAT+ cell population treated with 12.5 and 25 μ M were identical to those of controls (Table 2). Only when OGAT+ cells were exposed to 50 μ M TZM was a limited reduction in cell size and DNA content observed (Table 2). Fig. 2 illustrates the three-dimensional plots of a representative experiment using U937 clones. Cell size, correspond-

TABLE 2
Effects of TZM on DNA content and cell volume of OGAT– and OGAT+ leukemic cells

Cells were exposed to graded concentrations of TZM and after 72 hr analyzed by flow cytometry as described in Materials and Methods. Values represent the mean \pm standard error of three independent experiments. Data represent the percentage of cells characterized by a broad hypodiploid peak, typical of apoptosis. Values represent the mean \pm standard error of three independent experiments. Data represent the percentage of cells characterized by reduced cell size compared with untreated controls, as assessed by forward scatter analysis.

Cell line	TZM concentration	Cells with reduced DNA content	Cells with reduced volume
	μ M	%	
K562 OGAT–		5 \pm 1	5 \pm 1
	12.5	11 \pm 2	10 \pm 1
	25	25 \pm 1	25 \pm 3
	50	36 \pm 4	39 \pm 5
K562 OGAT+		5 \pm 2	3 \pm 2
	12.5	4 \pm 1	5 \pm 1
	25	5 \pm 2	7 \pm 1
	50	14 \pm 1	14 \pm 2
U937 OGAT–		5 \pm 2	3 \pm 2
	12.5	11 \pm 1	15 \pm 2
	25	28 \pm 2	29 \pm 3
	50	43 \pm 2	39 \pm 2
U937 OGAT+		3 \pm 2	2 \pm 3
	12.5	4 \pm 1	3 \pm 1
	25	5 \pm 1	5 \pm 2
	50	11 \pm 1	11 \pm 2

ing to forward light scatter, is plotted against the integral of the fluorescence (DNA content). The control cultures contained predominantly one peak with characteristic size distribution and fluorescence intensity (Fig. 2, A and C). OGAT– cells treated with 50 μ M TZM presented a broad peak corresponding to apoptotic cells characterized by reduced volume and hypodiploid DNA content (Fig. 2B). In contrast, only a small percentage of OGAT+ cells treated with the same drug concentration showed apoptotic changes (Fig. 2D).

It has been observed that apoptosis in K562 cells, unlike in that U937, occurs in the absence of double-strand breaks but in the presence of single-strand breaks (26). In this case, TdT-mediated b-dUTP nick-end labeling allows detection of DNA single-strand breaks associated with apoptosis (24, 26, 27). After treatment with TZM, 30–45% of the cells were labeled with b-dUTP (Fig. 3). On the other hand, no significant b-dUTP incorporation was detected in K562 OGAT– cells from untreated cultures (Fig. 3) or in OGAT+ K562 cells untreated or treated with TZM (data not shown). Simultaneous counterstaining of cells with PI and bivariate analysis of the data made it possible to correlate the incorporation of b-dUTP with cell position of the cell cycle. The incorporation of b-dUTP was limited predominantly to S and G2/M phases (Fig. 3). Similar results were obtained when OGAT+ or OGAT– U937 cells were treated with the same concentrations of TZM (data not shown).

OGAT expression prevents DNA fragmentation induced by TZM. DNA digestion to multiples of 180 bp by Ca²⁺/Mg²⁺-dependent endonuclease is considered a hallmark of apoptosis (28). Treatment of U937 OGAT– cells with increasing concentrations of TZM indeed resulted in the progressive appearance of a ladder-like pattern of DNA fragments consisting of multiples of \sim 180 bp as a result of internucleosomal DNA fragmentation (Fig. 4). On the contrary, TZM treatment of U937 OGAT+ cells did not induce internucleosomal DNA fragmentation (Fig. 4). During the process of apoptosis, degradation of genomic DNA starts with the excision of 50–300 kb fragments, which are then further digested to oligonucleosomal fragments. In K562 cells, apoptotic degradation does not proceed to completion and is arrested at the stage of 50-kb fragments (29). Therefore, to test whether TZM-induced apoptosis in K562 cells was associated with the generation of 50-kb DNA fragments and whether OGAT enzyme might prevent the appearance of this phenomenon, untreated and drug-treated controls and OGAT+ were analyzed by PFGE. The results, illustrated in Fig. 5 (left), show the appearance of DNA fragments in the range of 50 kb and higher-order fragments in K562 OGAT-deficient cells, which were more evident with the highest TZM concentrations. These fragments were absent in cells transduced with OGAT cDNA (Fig. 5, right). On the other hand, treatment of OGAT+ or OGAT– K562 cells with the inhibitor of topoisomerase II, VP16, resulted in the appearance of apoptotic DNA degradation in both lines, independent of the presence of the OGAT enzyme (Fig. 5).

BG abrogates resistance of OGAT-transduced leukemic cells to apoptosis induced by TZM. U937 OGAT+ cells were deprived of OGAT activity before treatment with TZM using BG. This specific OGAT inhibitor acts as a substrate of the OGAT protein, which, on interaction with BG, becomes inactivated and rapidly degraded. OGAT-depleted

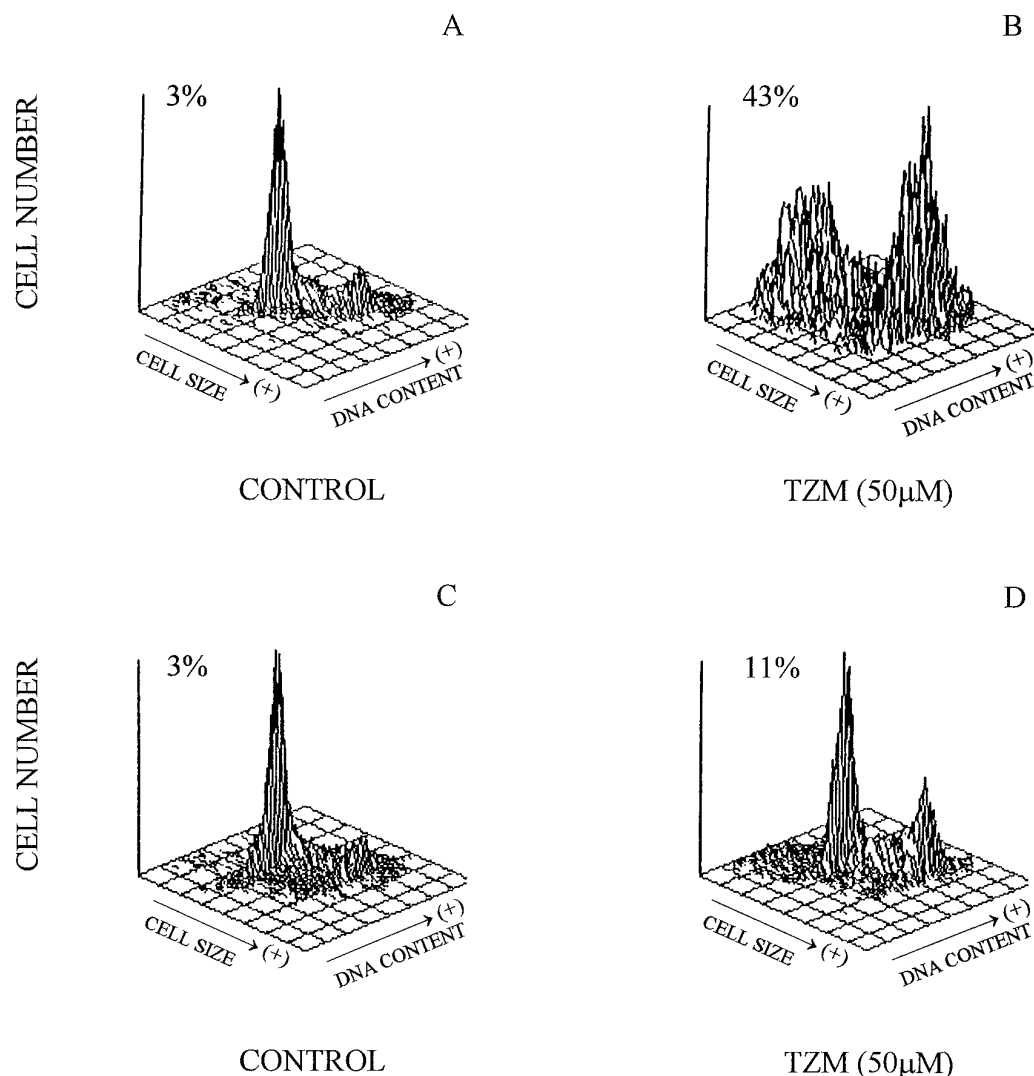


Fig. 2. Flow cytometry three-dimensional plots of untreated U937 OGAT- (A) or OGAT+ (C) clones and after treatment with 50 μ M TZM (B, OGAT-; D, OGAT+). Cells were processed for FACS analysis 72 hr after drug treatment, as described in Materials and Methods. X-axis, DNA content (PI-stained DNA). Y-axis, cell size. Z-axis, cell number. Percentages of cells with reduced volume and hypodiploid DNA content are indicated.

cells were subsequently exposed to 50 μ M TZM for 4 hr, washed, and cultured in the presence of BG to avoid recovery of OGAT activity due to resynthesis of new molecules of the enzyme. BG cell treatment reduced OGAT activity to undetectable levels (data not shown). The results of the flow cytometry analysis, illustrated in Fig. 6, show that depletion of OGAT increases cell susceptibility to apoptosis induced by TZM. These data were confirmed by the results of electrophoretic analysis of DNA fragmentation, which demonstrated the appearance of an apoptotic ladder only in cells exposed to BG and TZM (Fig. 7). The percentage of growth inhibition of cells exposed to TZM, untreated or pretreated with BG, was 10% and 64%, respectively.

Similar results were obtained when K562 OGAT+ cells were depleted of OGAT activity on treatment with BG and then exposed to TZM (data not shown).

BZ treatment increases U937 OGAT+ or OGAT- cell susceptibility to apoptosis induced by TZM. U937 OGAT+ cells were treated with 5 (data not shown) or 10 mM of the PADPRP inhibitor BZ (15) alone or together with 50 μ M TZM. Coexposure of OGAT+ cells to TZM and BZ caused a marked increase in the percentage of apoptotic cells compared with treatment with TZM alone. Fig. 8 illustrates the results obtained using 10 mM BZ. Similar results were ob-

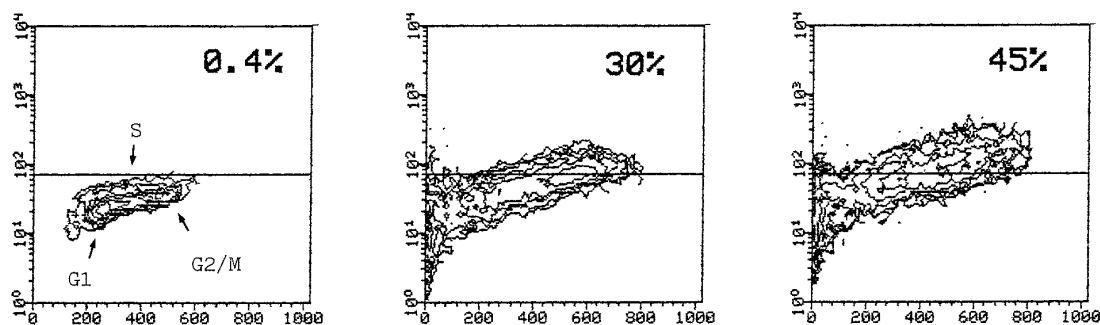
tained with OGAT- cells. Treatment of these cells with 50 μ M TZM induced apoptosis in 40% of cell population, whereas combined treatment with TZM and BZ (10 mM) induced apoptosis in 80% of the cell population. The percentage of apoptotic cells after exposure of OGAT- U937 to BZ was 36%.

Discussion

In this study, the human OGAT cDNA was transfected into leukemia clones that lack expression of alkyltransferase activity. Cell susceptibility to the cytotoxic and apoptotic effects of the antitumor agent TZM was evaluated. Cells of OGAT-transduced K562 or U937 leukemia showed increased resistance to TZM-mediated growth inhibition, with IC₅₀ values that were 4- or 10-fold higher, respectively, than those of cells transduced with a control virus. These results are consistent with those recently obtained by Wang *et al.* (30) in a murine model.

On treatment with TZM concentrations capable of inducing apoptosis in OGAT-deficient U937 or K562 cells, OGAT-transduced lines failed to show the appearance of DNA fragmentation patterns typical of the apoptotic process. In the case of U937 OGAT-deficient cells, TZM induced DNA cleavage into 180-bp oligonucleosome fragments, as demonstrated

b-dUTP incorporation by TdT
(green fluorescence)



DNA CONTENT (red fluorescence)

Fig. 3. Incorporation of b-dUTP by exogenous TdT into the DNA of K562 OGAT⁻ cells. Two-dimensional frequency-contour plots of DNA content (PI-stained DNA) versus b-dUTP, labeled with fluorescein isothiocyanate-conjugated avidin, incorporated by TdT. Cells were exposed to medium only (left) or 25 μ M (middle) or 50 μ M (right) TZM for 4 hr, washed, and cultured for 72 hr. Cells with green fluorescence above the line were considered positive for the TdT assay; percentages are indicated. Results are from one of three experiments. The mean values of three independent experiments were control, 0.4 ± 0.2 ; TZM (25 μ M), 26 ± 5 ; and TZM (50 μ M), 42 ± 3 .

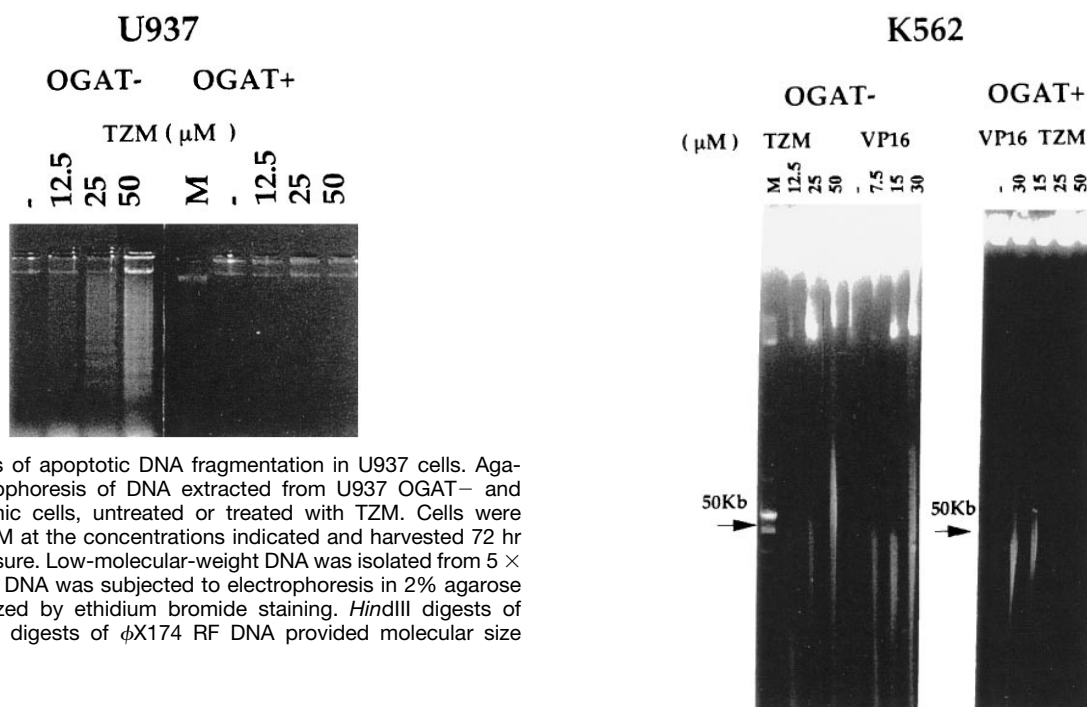


Fig. 4. Analysis of apoptotic DNA fragmentation in U937 cells. Agarose gel electrophoresis of DNA extracted from U937 OGAT⁻ and OGAT⁺ leukemic cells, untreated or treated with TZM. Cells were treated with TZM at the concentrations indicated and harvested 72 hr after drug exposure. Low-molecular-weight DNA was isolated from 5×10^6 cells. Then, DNA was subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. *Hind*III digests of λ DNA and *Hae* digests of ϕ X174 RF DNA provided molecular size markers (M).

by the results of conventional gel electrophoresis. At the same drug concentrations, U937 OGAT-proficient cells showed the DNA ladder only after OGAT depletion by BG, a specific inhibitor of the enzyme.

In OGAT-deficient K562 cells, TZM induced apoptosis as demonstrated by the results of PFGE analysis. In fact, in this cell line, apoptosis occurs predominantly through the generation of high-molecular-weight DNA fragments, without detectable internucleosomal fragmentation (29). Accordingly, it has been shown that a number of leukemia cell lines produce DNA fragments of ~ 50 kb during the process of chemotherapy-induced apoptosis (27). These large DNA fragments would arise from degradation of chromatin loops and higher-

Fig. 5. Analysis of apoptotic DNA fragmentation in K562 cells. PFGE analysis of high-molecular-weight DNA extracted from OGAT⁻ K562 (left) or OGAT⁺ K562 (right) cells treated with the concentrations of TZM or VP16. Cells were exposed to TZM for 4 hr or to VP16 for 1 hr and, after drug removal, incubated in CM for 72 hr. At the end of incubation, cells (2×10^6) were harvested, and cell pellets were embedded in agarose. Plug preparation and PFGE are described in Materials and Methods. M, Low-range PFGE molecular weight marker (New England Biolabs, Beverly, MA).

order structures (29). Moreover, OGAT-deficient K562 cells treated with TZM showed cell shrinkage and reduced DNA content (both are typical of apoptosis), as demonstrated by flow cytometry. Drug-treated cells also showed TdT-mediated

U937 OGAT+

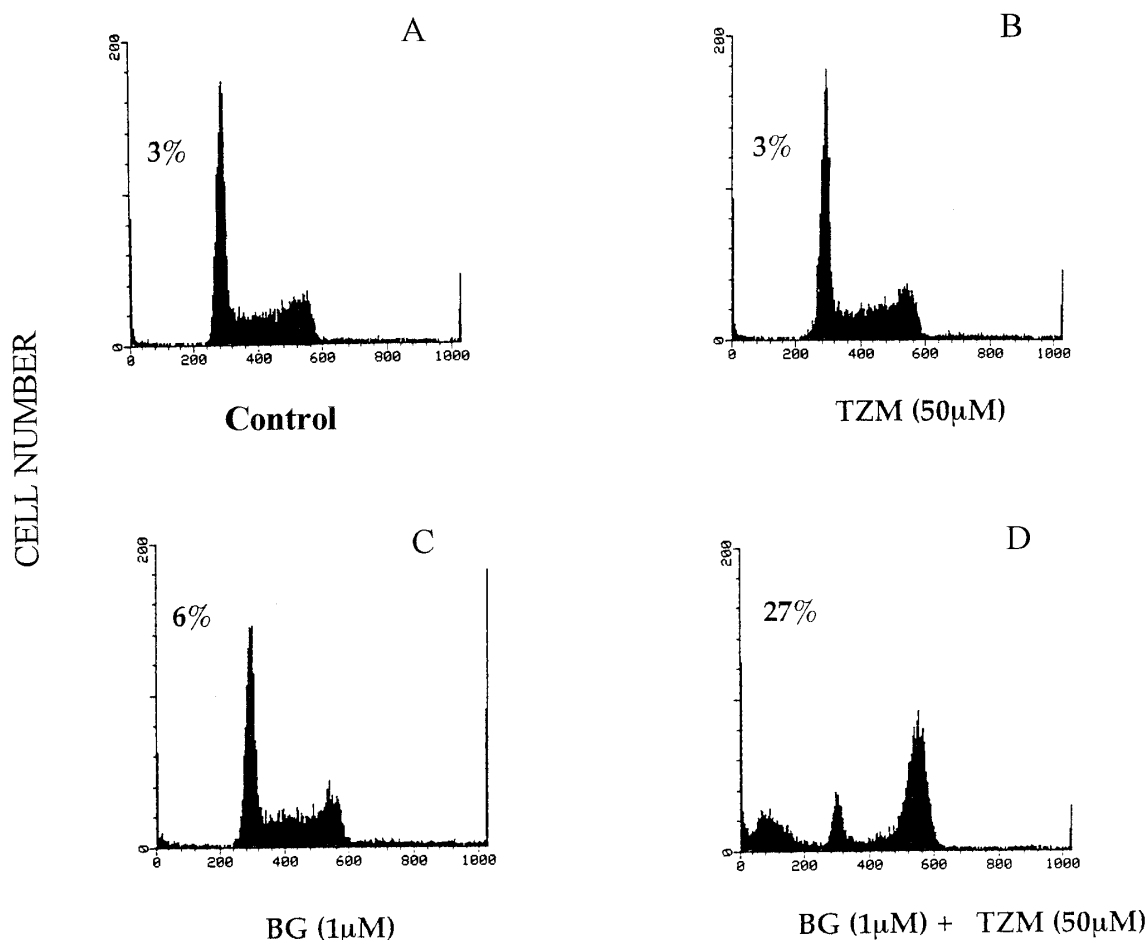


Fig. 6. Flow cytometry analysis of TZM-treated OGAT+ U937 cells after OGAT depletion by BG. A, Untreated cells. B, Cells treated with 50 μ M TZM. C, Cells treated with 1 μ M BG. D, Cells pretreated with BG and then exposed to 50 μ M TZM. Percentage of growth inhibition of cells exposed to TZM, untreated or pretreated with BG, was 10 and 64%, respectively. Results are from one of three experiments.

ated incorporation of labeled nucleotides. TdT assay has recently been considered capable of specifically detecting drug-treated cells that couple DNA damage to apoptotic response, even in cells that do not display DNA fragmentation to 180-bp fragments (27). Conversely, OGAT-proficient K562 cells failed to show morphological and biochemical apoptotic changes after treatment with TZM, as demonstrated by the results of flow cytometry, TdT assay, or PFGE. BG pretreatment of these cells resulted in down-regulation of OGAT levels. In this case, DNA fragmentation occurred on exposure to the triazene.

This is the first demonstration that methyl adducts at the O⁶ position of guanine, which are specifically repaired by OGAT, represent the DNA lesions responsible for induction of apoptosis by TZM.

Eastman *et al.* (31) have proposed since 1990 that killing of cancer cells by chemotherapeutic agents involves, at least in part, triggering of an apoptotic process. The first step of DNA fragmentation during the process of apoptosis seems to be characterized by the appearance of single-strand breaks (29). The methylating agent TZM is capable of inducing DNA single-strand breaks due to the formation of methyl adducts at the O⁶ position of guanine (32). In particular, unrepaired

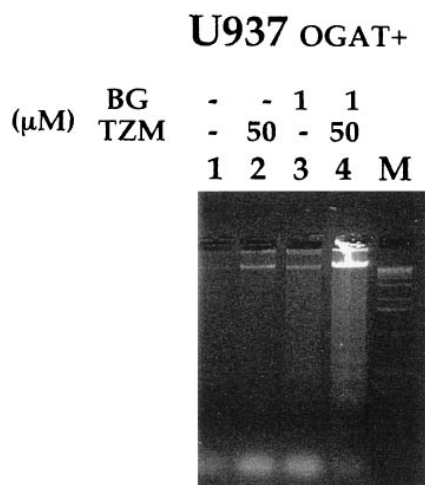


Fig. 7. Inhibition of OGAT activity by BG increases apoptotic effects of TZM. DNA gel electrophoresis of U937 OGAT+ clones, untreated or pretreated with BG, before exposure to TZM. Low-molecular-weight DNA isolated from untreated or drug-treated cells was subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. *Hind*III digests of λ DNA and *Hae* digests of ϕ X174 RF DNA provided molecular size markers (M).

U937 OGAT+

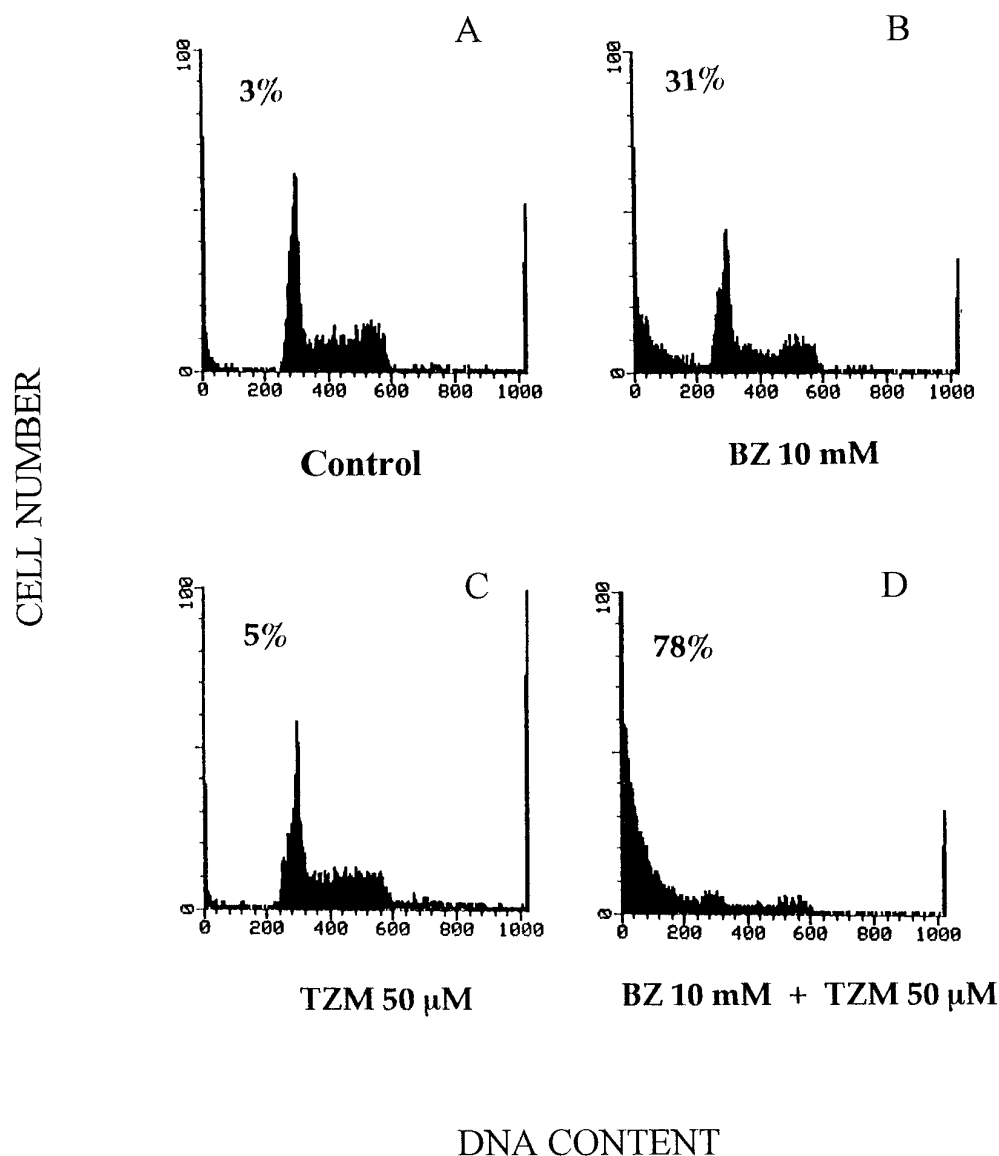


Fig. 8. Effect of BZ treatment on U-937 OGAT+ cell susceptibility to apoptosis by TZM. Untreated (A) or 10 mM BZ-treated cells (B) were exposed to 50 μ M TZM (C and D, respectively) and processed for FACS analysis 72 hr after drug treatment. Percentage of cells that exhibit the hypodiploid peak typical of apoptosis is shown. Results are from one of two experiments.

O⁶-methylguanine forms unstable pairs with thymine during the process of DNA replication. Thymine-O⁶-methylguanine mispairs are then recognized by the mismatch repair system (33). Inability of the mismatch repair to find the correct base complementary to O⁶-methylguanine results in unsuccessful repair, with the generation of DNA strand breaks (13). Thus, it is reasonable to hypothesize that DNA synthesis would be required for DNA damage to become evident. This is in agreement with the results of the TdT assay, which revealed the presence of DNA strand breaks in the S and G2/M phases of cell cycle in OGAT-deficient cells treated with TZM, as illustrated in Fig. 3.

A variety of studies indicate that DNA strand breaks activate PADPRP, which in turn binds strand interruptions and undergoes autoribosylation, synthesizing long and branched poly(ADP-ribose) chains (for a review, see Ref. 14). After

ribosylation, the PADPRP enzyme rapidly dissociates from DNA, allowing repair of DNA damage (34). It has been recently demonstrated that PADPRP inhibitors potentiate cytotoxicity of anticancer agents, including TZM, in murine leukemias (15, 35). In this report, we demonstrated that treatment of OGAT-proficient or -deficient U937 cells with BZ and TZM results in increased apoptosis. Even though further studies with more specific and potent PADPRP inhibitors are required, it is tempting to speculate that PADPRP might be involved in coordinating the repair of DNA lesions, different from O⁶-methylguanine, induced by TZM.

The products of a number of genes have been shown to be involved in the control of apoptosis. In particular, bcl-2 protein has been described as preventing chemotherapy-induced apoptosis in leukemic lines (36). In the current model, no

differences were observed in the expression of bcl-2 between OGAT-proficient and -deficient clones from both cell lines (K562 OGAT⁻ or OGAT⁺, 12% \pm 5; U937 OGAT⁻ or OGAT⁺, 30% \pm 6) as assessed by flow cytometry. Moreover, TZM treatment of K562 and U937 cell lines does not modulate the expression of bcl-2 protein (17).

Among negative regulators of the apoptotic process, the chimeric bcr-abl protein seems to be responsible of the well-documented resistance of chronic myelogenous leukemias (e.g., K562 cell line) to apoptosis by chemotherapeutic drugs (for a review, see Ref. 37). In apoptosis induced by TZM, bcr-abl does not seem to be involved in leukemic cell resistance to this agent, and reduction of bcr-abl protein levels, with antisense oligonucleotides treatment, did not affect apoptosis induced by TZM (data not shown).

Positive regulators of apoptosis include p53 protein, which participates in genome surveillance and DNA repair. One of the functions of p53 in cell cycle arrest at the G1/S boundary is to allow repair of damaged DNA before DNA replication or induction of apoptosis, when DNA damage is too severe. Expression of wild-type p53 in p53-deficient tumor cell lines renders them more susceptible to induction of apoptosis by radiation or DNA-damaging chemotherapeutic drugs (38). In the case of TZM-induced apoptosis, p53 does not seem to be required for the triggering of the process because both U937 and K562 cells possess a mutated p53 gene (39–40).

In conclusion, this study showed that methyl adducts at the O⁶ position of guanine are required for induction of apoptosis by TZM. Inhibition of OGAT or PADPRP increases the susceptibility of OGAT-proficient leukemia cell lines to TZM-induced apoptosis, thus suggesting new strategies for the treatment of relapsed or resistant acute leukemias.

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