Role of Wild-Type p53 on the Antineoplastic Activity of Temozolomide Alone or Combined with Inhibitors of Poly(ADP-Ribose) Polymerase

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Accepted for publication January 9, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The DNA repair enzyme O6-alkylguanine DNA-alkyltransferase (OGAT) and a deficient mismatch repair system play a critical role in the resistance to chemotherapeutic agents that generate adducts at the O6-position of guanine. However, DNA adducts different from O6-methylguanine might be also involved in cytotoxicity induced by methylating agents. Because the loss of p53 function is generally associated with tumor cell resistance to anticancer chemotherapy, we have investigated whether wild-type p53 might affect chemosensitivity of leukemia cells endowed with high OGAT levels to the methylating agent temozolomide (TZM). The effect of poly(ADP-ribose) polymerase (PADPRP) inhibition, which potentiates the cytotoxic effects of O6-methylguanine and N3-methylguanine, was also assessed in OGAT-proficient cells, either susceptible or tolerant to O6-methylguanine. OGAT-proficient and p53 null HL60 cells were transfected with the human p53 cDNA (p53+ cells). Treatment with TZM concentrations not toxic for the cells transduced with the control vector (p53- cells), induced apoptosis in p53+ cells.

Inactivation of p53 is a frequent event in development of human malignancies (Hollestein et al., 1991, 1994). Wild-type p53 is in fact a critical element in suppressing cell proliferation in response to DNA-damaging agents, either inducing G1 arrest or activating programmed cell death (reviewed in Hansen and Oren, 1997). Loss of p53 function leads to genomic instability and tumor cell resistance to anticancer therapy, including chemotherapeutic drugs and radiation (Livingstone et al., 1992; Yin et al., 1992; Lowe et al., 1994).

Wild-type p53 is also involved in activating DNA repair either directly or through transactivation of other genes (reviewed in Ko and Prives, 1996; Marx, 1994). In particular, p53 appears to affect the rate and efficiency of excision repair system (Wang et al., 1995a). In contrast, it has been recently demonstrated that wild-type p53 suppresses transcription of OGAT (Harris et al., 1996). This repair enzyme removes the alkyl adducts on DNA at the O6 position of guanine (for a review see Pegg et al., 1995). Alkyl adducts are generated by monofunctional methylating and chloroethylating agents. Killing by chloroethylating agents involves the formation of DNA interstrand cross-links, which are produced by intramolecular rearrangement of O6-chloroethylguanine adducts (Tong et al., 1982; Jlang et al., 1989). Conversely, the mechanism of cytotoxicity by methylating compounds is likely to

ABBRERVIATIONS: OGAT, O6-alkylguanine DNA-alkyltransferase; TZM, temozolomide; PADPRP, poly(ADP-ribose) polymerase; p53+, HL60 cells transfected with the human wild-type p53 cDNA; p53-, HL60 cells transfected with the control vector pLNSX; BZ, benzamide; ABZ, 3-amino-benzamide; BG, O6-benzylguanine; CM, complete medium; VP16, etoposide; ICE, interleukin-1β converting enzyme; O.D., optical density; PI, propidium iodide; glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
involve an inappropriate processing of O\textsuperscript{6}-methylguanine during DNA replication by the mismatch repair system, which generates DNA strand breaks (Karran and Bignami, 1992). Thus, high levels of OGAT and the loss of components of mismatch repair system, that involves long patch DNA repair, are the main factors responsible of tumor cell resistance to O\textsuperscript{6}-methylating agents (Liu et al., 1996; Karran and Bignami, 1994; Kat et al., 1993).

The PADPRP enzyme is activated in response to agents that cause DNA strand breaks (Lindahl et al., 1995). 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. This enzyme has been defined as one of the substrates cleaved by the ICE-like proteases during the process of apoptosis (Tewari et al., 1995).

Programmed cell death is characterized by different types of DNA fragmentation, such as degradation of DNA into oligonucleosome sized or large DNA fragments (50-300 Kb), and cleavage due to single strand breaks (for a review see Kumar and Lavin, 1996). Therefore, taking into account the role of PADPRP in DNA repair, it is conceivable to hypothesize that proteolysis of PADPRP during apoptosis would avoid the activation of this enzyme by DNA fragmentation, which would lead to repair under conditions where DNA should be degraded.

Recently, it has been shown that PADPRP inhibitors have the potential to act as resistance modifiers when used in combination with chemotherapeutic agents (Griffin et al., 1995). A previous study indicated that combined treatment with TZM and BZ, an inhibitor of PADPRP (Griffin et al., 1995), results in overall increase of apoptosis in either OGAT-proficient or OGAT-deficient leukemia cells (Tentori et al., 1997). Furthermore, it has been demonstrated that PADPRP inhibitors might enhance methylating agent activity also in tumor cells devoid of a functional mismatch repair system (Wedge et al., 1996). Thus, it was speculated that PADPRP might be involved in coordinating the repair of DNA adducts, different from O\textsuperscript{6}-methylguanine, induced by TZM. In fact, interaction of methylating compounds with DNA leads to the formation of adducts also at the N\textsuperscript{7} of guanine, N\textsuperscript{3} of adenine and other DNA base positions that are repaired by the base excision repair pathway (Barnes et al., 1993). The different types of modified bases are cleaved by specific glycosylases. Once the base is removed the further repair of the apurinic site requires the coordinate intervention of endonucleases, phosphodiesterases, DNA polymerases and ligases. In the absence of an efficient repair, depurination of 7-methylguanine or 3-methylguanine is followed by the generation of DNA strand breaks.

Methylating compounds include chemotherapeutic drugs such as dacarbazine, and its in vitro active derivative TZM, which is currently under phase II clinical study (Woll et al., 1995; Bleehen et al., 1995). Dacarbazine is active against melanomas, sarcomas and lymphomas and has shown a therapeutic potential for the treatment of acute nonlymphoid leukemias relapsed or refractory to conventional treatment (Franchi et al., 1992; D’Atri et al., 1995). Differently from dacarbazine, TZM does not require metabolic activation, has limited bone marrow toxicity and has demonstrated promising clinical activity in the treatment of gliomas and melanomas (Newlands et al., 1992). Recently, we have shown that TZM treatment of leukemia cell lines (i.e., U937 and K562) with low basal levels of OGAT is followed by the induction of apoptosis (Tentori et al., 1995). Moreover, transfection of these cell lines with the human OGAT cDNA reduced tumor cell sensitivity to the cytotoxic and apoptotic effects of this compound (Tentori et al., 1997). Down-modulation of OGAT by BG of OGAT-transfected lines or of HL60 cells, which express high levels of OGAT activity, restored leukemia cell sensitivity to apoptosis induced by TZM (Tentori et al., 1995 and 1997). In this model the contribution of a functional p53 to apoptosis induced by TZM could not be explored, since both U937 and K562 cells possess a mutated p53 gene (Dou and An, 1995; Bedi et al., 1995) and it has recently been demonstrated that p53 mutants might interfere differently with normal p53 function, depending on the type of mutations (Pocard et al., 1996).

The aim of our study was to investigate the influence of wild-type p53 on apoptosis induced by TZM. Moreover, the contribution to TZM-mediated cytotoxicity of DNA lesions different from those generated by the inappropriate processing of O\textsuperscript{6}-methylguanine, has been explored. The human p53 cDNA was transfected in p53 null HL60 cells (Wolff and Rotter, 1985) resulting in a stable expression of low levels of p53 protein. The data indicate that the presence of wild-type p53 increases apoptosis by TZM even in cells naturally resistant to O\textsuperscript{6}-methylating agents, due to high levels of OGAT activity. Inhibition of PADPRP potentiated apoptosis induced by TZM in p53\textsuperscript{+} and p53\textsuperscript{-} cells with high levels of OGAT activity and in leukemia cells characterized by a mutated form of p53 gene and tolerant to O\textsuperscript{6}-methylguanine adducts.

Materials and Methods

Cell lines. Three human leukemia cell lines were used: HL60, a promyelocitic cell line (ATCC CCL 240); Daudi, a Burkitt lymphoma cell line (ATCC CCL 213); Jurkat, a T-cell leukemia line (ATCC CRL 8136).

Cells were cultured at 37°C in 5% CO\textsubscript{2} humidified atmosphere in RPMI-1640 (GIBCO, Paisley, Scotland, UK) supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (GIBCO), 2 mM L-glutamine, and antibiotics (Flow Laboratories, McLean, VA) (hereafter referred to as CM). Clones were obtained by limiting dilution.

The amphotropic packaging cell line PA317 and NIH3T3 cells were obtained from Dr. J. Schlom (National Cancer Institute, NIH, Bethesda, MD). PA317 and NIH3T3 cells were cultured in DMEM (GIBCO) supplemented with 10% FCS and L-glutamine.

Reagents. TZM was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). [\textsuperscript{3}H]-methylated-DNA, to be used as substrate for determination of OGAT activity, was prepared using [\textsuperscript{3}H]-methyl-N-nitrosourea (specific activity 19.2 Ci/mmol, Amersham International Plc, Amersham, UK) (Margison et al., 1985). BG was synthesized and kindly gifted by dr. L. Lassiani (Institute of Pharmacological Chemistry, University of Trieste, Trieste, Italy). Etoposide (VP16), BZ and ABZ were purchased from Sigma Chemical Co. (St. Louis, MO). TZM, BZ and ABZ stock solutions were prepared by dissolving the drugs in RPMI-1640, whereas BG was dissolved in ethanol.

The antibodies used in this study were as follows: anti-p53 (Ab-2) monoclonal antibody (Oncongene Sciences, Cambridge, MA), anti-PADPRP polyclonal antibody (Boehringer-Mannheim, Monza, Italy), anti-bel-2 (clone 124) monoclonal antibody (Dako, Gentebrugge, Belgium), anti-bax (P-19) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The anti-human OGAT polyclonal anti-
serum was generated in our laboratory, using the fusion protein glutathione-S-transferase-OGAT to immunize New Zealand rabbits.

**Transfection and transduction of p53 cDNA.** A HindIII fragment, encompassing the human p53 cDNA (Lamb and Crawford, 1986) was cloned into the retroviral vector pLNSX (Miller and Rosman, 1989). After DNA sequencing by the chain termination method using Sequenase version 2.0 (US Biochemical, Cleveland, OH), these constructs or the vector pLNSX were transfected into the PA317 packaging cell line, using the calcium phosphate precipitation procedure. Stable virus producing cell lines were generated following selection of the mass culture with 1 mg/ml G418. The apparent virus titers were determined on murine NIH3T3 cells by G418 selection and ranged between 10^4 and 4 x 10^5 colony forming units/ml. HL60.12 clone, in the exponential phase of growth, was transduced by replacing culture medium with a 1:1 mixture of p53 recombinant, or control virus producing PA317 cells and fresh CM added with polybrene (8 μg/ml). Two days after transduction, cells were G418 selected (0.5 mg/ml). Control or not transduced cells died at this antibiotic concentration.

**Drug treatment and cell growth evaluation.** Cell suspensions containing 1 x 10^6 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 ranging between 50 and 200 μM. Cell growth was evaluated in terms of viable cell count every 24 hr for 3 days. Cells were manually counted using a haemocytometer and cell viability was determined by trypsin blue exclusion test or by flow cytometry analysis of cells stained with PI. All determinations were made in quadruplicate. Depletion of OGAT activity was obtained by treating tumor cells (1 x 10^6/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 mM) or TZM exposure. BG was then added again every 24 hr. Inhibition of probe was a 572-bp cDNA fragment obtained by RT-PCR using total contamination. cDNA was synthesized by incubating 2 μg of total RNA derived from Daudi cells and the primers described above. The OGAT probe was a PCR-derived cDNA probe obtained after reverse transcription of the RNA from Molt-4 cells (Tentori et al., 1995). After washing with 0.1 x SSC (10 mM sodium chloride, 1.5 mM sodium citrate) at room temperature for 30 min, the blotted membranes were exposed to x-ray films (Kodak, Rochester, NY) at -80°C. Bidimensional densitometry of the immunoblot was performed using a BioRad (Richmond, CA) scanning apparatus (Imaging densitometer, GS-670).

**Assay for OGAT activity.** This assay was performed as previously described (Morten and Margison, 1988), with minor modifications. Briefly, cells (2 x 10^9) were lysed in 0.5 ml of lysis buffer containing 0.5% CHAPS, 50 mm Tris-HCl pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, 1% glycerol, 2 μg/ml leupeptin and freshly added 1 mM PMSF. The lysates were then microfuged at 15,000 rpm at 4°C 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 a calf thymus DNA, which had been previously methylated by reaction with N,N,N′-trimethyl-N-nitrosourea. OGAT activity was expressed in terms of fmol/10^9 cells.

**Flow cytometry analysis.** Cells from cultures were washed with phosphate-buffered saline and fixed in 7% ethanol at -20°C for 18 hr. The centrifuged pellets were resuspended in 1 ml of hypotonic solution containing PI (50 μg/ml), 0.1% sodium citrate, 0.1% Triton-X and RNase (10 μg/ml). Cells were incubated in the dark, at 37°C for 30 min. Data collection was gated using forward light scatter and side light scatter to exclude cell debris and cell aggregates. PI fluorescence was measured on a linear scale using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Apoptotic cells are represented by a broad hypodiploid peak, which is easily discernible from the narrow peak of cells with diploid DNA content in the red fluorescence channel (Nicoletti et al., 1991). All data were recorded and analyzed using Lysis II software (Becton Dickinson). Cell-Fit software (Becton Dickinson) was used for cell cycle analysis.

**Western blotting.** Cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4 and freshly added 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), an aliquot saved for protein concentration determination (using BioRad protein assay solution and bovine serum albumin as standard), and the rest immediately boiled in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). One hundred μg of proteins per sample were electrophoresed on 10% SDS-polyacrylamide gels. Afterwards, proteins were transferred on Hybond-C membrane (Amersham). For p53, bel-2 and bax, immunodetections were carried out using Bethlinger-Mannheim chemiluminescence western blotting kit, according to manufacturer’s instructions. Filters were exposed to X-OMAT AR autoradiographic films (Kodak) for periods of time ranging between 5 sec and 20 min. For PADPRP immunodetection, filters were blocked with 3% non-fat dry milk in TBST (20 mM Tris-HCl pH 8.0, NaCl 0.9%, 0.03% Tween 20) and then incubated with PADPRP monoclonal antibody (5 μg/ml) in the same blocking buffer for 1 hr. After washing with TBST, immunocomplexes were visualized using an alkaline phosphatase-coupled anti-mouse IgG antibody and the Protoblot color development system (Promega Biotech, Madison, WI). Densitometric analysis of the immunoblots was performed as described above.

**Results**

Establishment of HL60 cells expressing low levels of wild-type p53. The role of wild-type p53 protein in modulating apoptosis by TZM has been studied by transfecting p53 cDNA in HL60 cell line, which contains gross deletions in both alleles and therefore it can be considered null for p53. Amphotropic PA317 cells were transfected either with the
construct pLNSX/p53 or with the pLNSX control vector. Supernatants from virus producer PA317 cells were used to infect a clone obtained from HL60 cell line. To determine p53 expression in transfected cells, G418 selected cells were analyzed for the presence of p53 transcript by RT-PCR, as described in “Materials and Methods.” The p53 transcript was detected in HL60 cells transfected with the human p53 cDNA (fig. 1A, lane 1) as well as in Daudi cells that express a mutated form of p53 (fig. 1A, lane 3) (Gaidano et al., 1991), whereas it was absent in cells transfected with the pLNSX vector (fig. 1A, lane 2). Amplification of the GAPDH cDNA was obtained in all samples (fig. 1A). The presence of the p53 transcript was further confirmed by the results of northern blot analysis which indicated the presence of virus-derived p53 transcript in p53 transfected HL60 cells (fig. 1B, lane 1).

The expression of exogenous p53 protein in p53+ cells was evaluated by Western blot analysis. p53 transfected cells expressed low levels of p53 protein, although pLNSX transfected controls did not (fig. 1C).

To test whether transfected cells contained a functional p53 protein capable of inducing arrest of the cell cycle at the G1/S boundary, cells were exposed to x-rays. Low doses of x-rays were used since G1 arrest predominates at doses lower than 1 Gy, whereas G2 arrest becomes more prominent at higher doses (Kastan et al., 1991). Irradiation of p53 transfected cells to nonlethal doses of x-rays resulted in a 50% (0.5 Gy) and 30% (1 Gy) decrease of cells entering S phase of the cell cycle when irradiated cells were compared to untreated controls. These results suggested that cells that were in G1 during the time of DNA damage did not progress to S phase.

On the contrary, HL60 p53- control or Jurkat cells, which harbor in their genome a mutated form of the p53 gene (Iwamoto et al., 1996), did not show a substantial decrease in the percentage of cells in the S phase, with respect to untreated lines (0 and 5% reduction with 0.5 and 1 Gy of x-ray exposure in both cell lines). None of the cell lines tested underwent apoptosis under these experimental conditions (data not shown).

Wild-type p53 transduction increases apoptosis induced by TZM in OGAT-proficient HL60 cells. Loss of p53 function is generally associated with inability of the cell to undergo apoptosis when cells are exposed to serum deprivation or DNA damaging agents. In fact, in our model, wild-type p53+ cells were more sensitive than p53- cells to apoptosis that follows serum deprivation of cell culture or treatment with the anticancer agent VP16 (data not shown).

To investigate whether p53 transduction might increase sensitivity to apoptosis induced by TZM, untreated or drug-treated HL60 p53- and p53+ cells were analyzed using flow cytometry, which allows to examine cell cycle and the decrease in fluorescence intensity of the DNA, characteristic of the apoptotic process.

In p53- cells, 72 hr after the exposure to 100 or 200 µM TZM, DNA content was similar to that of controls (fig. 2, top). On the contrary, the percentage of p53+ cells with hypodiploid DNA peak increased progressively with increasing concentrations of the agent (fig. 2, bottom). These data were confirmed by analysis of DNA fragmentation using conventional agarose gel electrophoresis (data not shown).

Western blot analysis of PADPRP protein in untreated or TZM-treated p53- or p53+ cells showed the presence of the 25-kDa cleaved product only in p53+ cells treated with 100 or 200 µM TZM (fig. 2, right panel). This protein is generated by proteolysis of PADPRP induced by ICE-like proteases during apoptosis and contains the amino-terminal DNA-binding domain of the PADPRP molecule (Tewari et al., 1995; Kaufmann et al., 1993).

Low levels of wild-type p53 expression do not affect OGAT gene transcription or activity. A previous study described that p53 overexpression can downregulate the OGAT promoter (Harris et al., 1996). Therefore, to verify whether increased apoptotic effects of temozolomide in p53+ OGAT-proficient cells might be due to the reduction of OGAT transcription, Northern, Western blot analysis and evaluation of OGAT activity were performed. The results, illustrated in figure 3, indicate that p53 transfected or control HL60 cells expressed comparable levels of OGAT transcript and protein (fig 3A and B). Both cell lines showed similar basal levels of OGAT activity (fig. 3). Wild-type p53+ and p53- cells were also assayed for OGAT activity before and 24, 48 or 72 hr after treatment with increasing concentrations of TZM. After 24 hr, TZM treatment induced a parallel decrease of activity in both cell lines (fig. 3C). Moreover, resynthesis of new OGAT protein molecules occurred in a similar fashion in both lines, as indicated by the values of OGAT activity obtained at 48 and 72 hr (data not shown).

Effect of BG on HL60 transfected cells treated with TZM. Cells were deprived of OGAT activity before treatment with TZM using BG. This specific OGAT inhibitor acts as a substrate of the OGAT protein that, upon interaction with BG, becomes inactivated and rapidly degraded (Dolan et al., 1990). OGAT-depleted cells were subsequently exposed to increasing concentrations of TZM (50-200 µM) and cultured in the presence of BG to avoid recovery of OGAT activity, due to resynthesis of new molecules of the enzyme. Treatment of the cells with BG reduced OGAT activity to undetectable levels (data not shown). The results illustrated in figure 4 show that depletion of OGAT activity in p53- cells augmented cell susceptibility to cytotoxicity induced by TZM. Conversely, inhibition of OGAT activity in p53+ cells did not...
increase the cytotoxic effects of the agent. Moreover, it is noteworthy that TZM treatment inhibited cell growth more efficiently in p53+ cells than in either OGAT-proficient or OGAT depleted p53- cells (fig. 4). Similar results were obtained when apoptosis was evaluated (data not shown).

Effect of wild-type p53 transfection on bax and bcl-2 protein expression. To investigate whether wild-type p53 protein might affect the expression of gene products that are involved in apoptosis downstream of p53, we evaluated the relative expression of bax and bcl-2 in p53- or p53+ cells and in O6-methylguanine tolerant cells. In a

Fig. 2. Expression of wild-type p53 increases apoptosis by TZM. Left panels, Flow cytometry analysis of DNA content. Flow cytometry profiles of p53- (top) or p53+ (bottom) cells untreated or exposed to 100 and 200 μM TZM. Cells were processed for FACS analysis 72 hr after drug treatment. Percentages of cells with hypodiploid DNA content are indicated. Right panel, Western blot analysis of PADPRP protein cleavage. Lane 1, untreated p53+ cells; lane 2, p53+ cells treated with 100 μM TZM; lane 3, p53+ cells treated with 200 μM TZM; lane 4, p53- cells treated with 200 μM TZM; lane 5, p53- cells treated with 100 μM TZM; lane 6, untreated p53- cells. The presence of the 25-kDa cleaved product, detected only in p53+ cells treated with TZM is indicated by an arrow.

Fig. 3. Low levels of wild-type p53 do not affect OGAT gene expression. A, Northern blot analysis of OGAT transcript. Equal amounts of total RNA samples (15 μg of RNA) from each cell line (lane 1, p53+ cells; lane 2, p53- cells), were separated by gel electrophoresis and transferred to nylon membrane. The OGAT transcript was identified using a 600 bp [32P]-labeled OGAT cDNA probe. O.D., expressed in arbitrary units, were as follows: p53+: 4.2 O.D.; p53-: 3.9. O.D. B, Western blot analysis of OGAT protein expression. Immunodetection was performed using anti-OGAT polyclonal antiserum. Lane 1, p53+ cells; lane 2, p53- cells. Filter was exposed to autoradiographic film for 30 sec. The results of densitometric analysis were the following: p53+: 3.1 O.D.; p53-: 2.9 O.D. Basal OGAT activities of p53+ and p53- HL60 cells were 62 ± 4 and 56 ± 4 fmol/10^6 cells, respectively. C, Evaluation of OGAT activity in p53+ (C) or p53- cells (O) untreated or exposed to 50, 100 and 200 μM TZM for 24 hr. Data represent the mean of three determinations. Bars: S.E.
previous study we demonstrated that treatment of either OGAT-proficient or -deficient cells with the PADPRP inhibitor BZ and TZM resulted in increased apoptosis (Tentori et al., 1997). This result suggested that PADPRP might be involved in coordinating the repair of DNA lesions induced by TZM different from the methylation of the O6 position of guanine. In order to test whether a wild-type p53 might potentiate the cytotoxic and apoptotic effects of the combined treatment with TZM and a PADPRP inhibitor, HL60 transduced cells were exposed to a BZ concentration devoid of cytotoxic or apoptotic activity in parental HL60 cells (fig. 5A). Treatment with BZ (5 mM) induced apoptosis only in cells transfected with wild-type p53 (fig. 5A). Coexposure of transduced cells to TZM and BZ caused a marked increase in the percentage of apoptotic cells compared to treatment with TZM alone in both lines (fig. 5A). Similar results were obtained with not transduced HL60 bulk population (fig. 5A). Similar results were obtained when TZM was associated with the PADPRP inhibitor ABZ (data not shown).

Tumor cell resistance to methylating compounds, including TZM, generally correlates with high levels of OGAT activity. However, recently it has been shown that a deficient mismatch repair system could represent another mechanism of resistance to methylating agents (Liu et al., 1996) and that the combination of a PADPRP inhibitor with BG potentiates TZM cytotoxicity (Wedge et al., 1996). To test whether BZ might affect TZM susceptibility of leukemia cells endowed with high OGAT levels and tolerant to O6-methylguanine lesions (Tentori et al., 1997), p53 null Jurkat cells, were exposed to either non-toxic (200 μM, 0% growth inhibition at 72 hr) or moderately cytotoxic (400 μM, 21% growth inhibition at 72 h) concentrations of TZM together with 5 mM BZ. The data illustrated in figure 5B show that in the presence of
BZ Jurkat cells become susceptible to apoptosis induced by TZM.

Perturbation of cell cycle progression in transduced cells after treatment with ABZ and TZM. Transduced cells were tested for cell cycle arrest at various times (24, 48 and 72 hr) after treatment with ABZ (5 mM) or TZM (200 μM) alone, or with the drug combination. The time course of the percentage of cells in G1 and in S phase are shown in figure 6. Both p53 or pLNSX transduced HL60 cells, untreated or treated with ABZ, showed a similar pattern of cell cycle distribution (fig. 6). The G1 percentage of wild-type p53 transduced cells exposed to the drug combination did not change during the first 48 hr and increased at 72 hr. In contrast, the S phase dramatically decreased during the period of observation, thus indicating that cells that are in G1 do not continue to enter S phase. On the contrary, in pLNSX transduced HL60 cells an initial drop in G1 percentage, was observed with no substantial changes in the percentage of cells in S phase, with respect to untreated or ABZ-treated cells. Both p53+ or p53- cells that were in S phase appeared to continue to progress to G2/M (data not shown). Exposure of wild-type p53 transduced cells to TZM induced a progressive decrease in the percentage of cells in G1 and only a slight increase in the percentage of cells in S (fig. 6) and G2/M phase (data not shown). TZM treatment of pLNSX transduced HL60 cells did not affect the growth rate and cell cycle distribution.

Discussion

Positive regulators of apoptosis include p53 protein, which participates in genome surveillance and DNA repair. One of the functions of p53 consists in cell cycle arrest at the G1/S boundary 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 (Banerjee et al., 1995).

To test whether the presence of wild-type p53 might modulate apoptosis induced by TZM, a clone from p53 null HL60 cells was infected with an amphotropic virus containing the human p53 cDNA or the control vector. A stable p53+ transfectant was obtained, which presented clearly detectable levels of virus-derived p53 transcript. However, the level of expression of the corresponding translational product was extremely low. Nevertheless, p53 expression in HL60 cells induced growth arrest, as indicated by the marked decrease of the percentage of cells in the S phase of the cell cycle, 20 hr after exposure to low doses of x-rays (≥1 Gy). A role for p53 protein in G1 arrest is consistent with previously published experiments demonstrating that transfection of wild-type p53 gene into various cell lines induces G1 arrest (Baker et al., 1990; Diller et al., 1990; Wang et al., 1995b).

Restoration of p53 function in p53-null cells increased susceptibility also to apoptosis by TZM. Actually, HL60 cell line is characterized by high levels of OGAT and therefore it is resistant to the cytotoxic and apoptotic effects of TZM concentrations with a clinical relevance (50 μM) (Newlands et al., 1992). Transfection of wild-type p53 in these cells rendered them susceptible to TZM concentrations that did not affect (50 μM) or slightly impaired (100-200 μM) the growth of cells transduced with the control vector.

Pretreatment of transduced lines with the specific OGAT inhibitor, BG, increased cell killing by TZM mainly in p53- cells. It could be hypothesized that differential sensitivity to TZM, could be due to differences in the basal levels of OGAT activity between p53- and p53+ cells. In fact, it has been demonstrated that overexpression of wild-type p53 can suppress transcription of the human OGAT gene (Harris et al., 1996). However, in our study the low levels of p53 protein did not affect OGAT protein expression and activity. Because repair of methylated DNA by OGAT is followed by inactivation of the enzyme, methylation agents indirectly decrease OGAT levels by generating O6-methylguanine DNA adducts that are repaired by the alkyltransferase (Laclal et al., 1996). Therefore, OGAT activity was also tested in p53+ or p53- cells after exposure to TZM. Treatment of both transduced lines with TZM equally down-regulated OGAT activity. Moreover, resynthesis of new OGAT protein molecules at 48 and 72 hr occurred in a similar fashion in both lines. These data suggested that the increase of TZM-mediated apoptosis in p53+ cells did not involve inhibition of the repair of O6-methylguanine adducts.

It has been reported that p53 activates transcription of death genes (bax) or repress the transcription of survival genes (bcl-2) (Miyashita et al., 1994; Miyashita and Reed, 1995). These two gene products have been implicated as mediators of p53-induced apoptosis (for a review see White, 1996). The results illustrated in our report indicated that the basal levels of bcl-2 protein were lower than those detected in control p53- cells. This raises the possibility that p53 transfection might have down-modulated bcl-2 expression. Thus, the resulting imbalance between bax and bcl-2 proteins might contribute to increase cell susceptibility to apoptotic signals.

A variety of studies indicate that DNA strand-breaks activate PADPRP, which in turn binds strand interruptions and undergoes autoribosylation (Lindahl et al., 1995). After ribosylation, the PADPRP enzyme rapidly dissociates from DNA and allows repair of DNA damage (Satoh and Lindahl, 1992). In vitro activation of PADPRP by methylating compounds, such as TZM, has been previously described (Tisdale, 1985). In addition, it has been demonstrated that PADPRP inhibitors potentiate cytotoxicity of anticancer agents, including TGZ (Griffith et al., 1995; Boulton et al., 1995; Tentori et al., 1997). Specifically, we demonstrated that BZ treatment of OGAT-proficient and OGAT-deficient cell lines resulted in a marked enhancement of apoptosis by TZM. In this report, we have shown that treatment of OGAT proficient either p53- or p53+ cells with BZ or ABZ and TZM resulted in increased apoptosis. Furthermore, PADPRP inhibition enhanced TZM susceptibility to apoptosis of Jurkat leukemia cells, which are endowed with high OGAT activity and possess a mutated p53 gene. Thus, treatment with PADPRP inhibitors allowed to increase tumor cell chemosensitivity to methylating compounds even in the presence of mutated p53, which is generally associated with reduced susceptibility to anticancer agents. Moreover, the possibility to enhance cytotoxicity induced by TZM in OGAT-proficient cells appears to be relevant especially when downmodulation of OGAT by BG cannot be achieved (Edara et al., 1996).

When tumor cells are mismatch repair deficient OGAT depletion by BG is ineffective in potentiating methylating
agents cytotoxicity (Wedge et al., 1996). We have previously demonstrated that treatment of Jurkat cells with BG inhibited their OGAT activity without increasing their sensitivity to the cytotoxic and apoptotic effects of TZM (Tentori et al., 1995). Thus, these cells show a methylation-tolerant phenotype that presumably derives from a deficiency in the mis-

![Graphs showing time course of cell cycle changes in HL60 transduced cell lines. Curves represent the evolution of the percentages of cells in G1 (left panels) and S-phase (right panels). Wild-type p53+ (●) and p53− cells (○) were treated with ABZ (5 mM), TZM (200 μM) or the combination of the two drugs. Untreated cells are also represented (CTR). Each time point represents the average of two independent experiments. Bars: S.E.](http://jpet.aspetjournals.org)
match repair pathways. Therefore it is conceivable to hypoth-
e-size that the BZ-mediated increase of apoptosis induced by TTM might be related to an inefficient repair of methyl
adducts at N7 and N3 positions of guanine.

The results of the analysis of cell cycle distribution in HL60 cells treated with the combination ABZ + TTM showed a G1 arrest only in wild-type p53 transduced cells, as evidenced by the marked and progressive decrease of S phase. Conversely, exposure of p53- cells to TTM was associated with a dra-
matic drop in the percentage of cells in G1 phase and a progressive increase in the percentage of cells in S and G2/M phase. These data indicate that the DNA lesions involved in the cytotoxicity associated with the drug combination of TTM and PADPRP inhibitors provoked cell cycle perturbations different from those induced by the exposure of cells to TTM alone. Therefore it is possible to speculate that PADPRP inhibitors might affect the repair of DNA adducts which are processed differently from O6-methylguanine, thus inducing a different pattern of cell cycle distribution.

Further studies are required to investigate whether the presence of a functional p53 in mismatch deficient cells might further improve the role of BZ in potentiating temozolomide activity.

In conclusion, these data indicate that the presence of wild-type p53 increases tumor cell killing by TTM in OGAT-
proficient cells. Furthermore, the results suggest a possible role of PADPRP inhibitors in potentiating TTM activity against acute leukemias, independently on OGAT or mis-
match repair systems and even in the presence of a mutated p53 gene.

Acknowledgments

The authors thank Dr. S. D’Atri for discussion, C. Mastrilli, G. Bonelli for their excellent technical assistance and are grateful to Dr. P. Ballerini for providing the human p53 cDNA.

References

Baker SJ, Markowitz S, Fearon ER, Willson JK and Volgelstein B (1990) Suppres-
sion of human colorectal carcinoma cell growth by wild-type p53. Science 240:912-
915.


Edara S, Kanugula AE, Goodrich K and Pegg AE (1996) Resistance of the human O6-alkylguanine-DNA alkyltransferase containing argon 160 to inactiv-


cer Res 56:2029-2032.


Iwamoto KS, Mizuno T, Ito T, Tsuaya N, Kysuzumi S, Seyama T (1996) Gain of function p53 mutations enhance alteration of the T-cell receptor following X-irra-
diation, independently of the cell cycle and cell survival. Cancer Res 56:5514-5517.


Lacal PM, D’Atri S, Orlande L, Bonmassar E and Graziani G (1996) In vitro inactivation of human O6-alkylguanine-DNA alkyltransferase by antitumor tria-
zene compounds. J Pharmacol Exp Ther 279:416-422.


Lindahl T, Satoh MS, Poirier GG and Klungland A (1995) Post-translational modi-
fication of poly(ADP-ribose) polymerase induced by DNA strand breaks. TIBS 20:405-411.

Liu L, Markovits S and Gerson SL (1996) Mismatch repair mutations override alkyltransferase conferance in colon xenografts but not to 1,3-bis(2-


MARGISSON GP, COOPER DP and BRENNAN J (1985) Cloning of the E Coli O6-
methylguanine and methylphosphoesther methyl transferase gene using a func-


Wedge SR, Porteus JK and Newland ES (1996) 3-Aminobenzamide and/or O6-benzylguanine evaluated as an adjuvant to temozolomide or BCNU treatment in cell lines of variable mismatch repair status and O6-alkylguanine-DNA-alkyltransferase activity. *Br J Cancer* 74:1030-1036.


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