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ORIGINAL ARTICLE

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Effects of single or split exposure of leukemic cells to temozolomide, combined with poly(ADP-ribose) polymerase inhibitors on cell growth, chromosomal aberrations and base excision repair components

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Abstract *Purpose*: To evaluate the antitumor activity of single versus split exposure of neoplastic cells to temozolomide (TZM) and poly(ADP-ribose) polymerase (PARP) inhibitor. *Methods*: A leukemic Jurkat cell line and freshly isolated leukemic blasts were used. Jurkat cells are resistant to O^6-methylguanine damage induced by TZM due to high levels of O^6-alkylguanine-DNA alkyltransferase and to a functional defect in the mismatch repair system. Cells were treated with 3-aminobenzamide or with NU1025 to inhibit PARP activity. TZM was added to cell cultures immediately after PARP inhibitors. The concentrations of TZM used were 62.5 μ*M* (corresponding to the peak plasma concentration in patients) or 125 μ*M*. *Treatment design*: Cells were treated with 125 μ*M* TZM plus PARP inhibitors (single exposure), or twice with 62.5 μ*M* TZM plus PARP inhibitors with an interval of 24 h between treatments (split exposure). Tumor cell growth, clastogenicity and base excision repair gene transcripts or enzymatic activity were evaluated. *Results*: The split exposure of Jurkat cells to TZM induced more pronounced and persistent growth inhibition and comparable chromosome damage in comparison with the single exposure. In addition, PARP inhibitors potentiated the cytotoxic effects induced by repeated treatment with TZM in fresh leukemic blasts. A marked decrease in X-ray repair cross-complementing 1 transcript and methylpurine glycosylase (MPG) transcript was detected in Jurkat cells subjected to the split exposure. In this case, a significant reduction in the corresponding enzymatic activity was also observed. *Conclusions*: Cytotoxicity induced by TZM and PARP inhibitors can be improved by a fractionated modality of drug treatment. The reduction in MPG transcript and function would presumably contribute to an increase in cell susceptibility to DNA damage induced by the methylating agent and PARP inhibitors.

Key words Temozolomide · Poly(ADPribose) polymerase inhibitors · Leukemic cells · Base excision repair

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Introduction

Temozolomide (TZM) is an orally administered methylating agent capable of crossing the blood-brain barrier and has a favorable toxicity profile [19]. These properties have indicated that TZM is a suitable candidate for a number of clinical studies. In particular, TZM has shown activity against solid tumors, such as high-grade glioma and melanoma [18, 20, 34]. In addition, preclinical and clinical studies have indicated that TZM might also be cytotoxic in tumor cells of hematopoietic origin [5, 9, 17, 22, 25, 26, 27]. The antitumor activity of TZM has been mainly attributed to the formation of O^6-methylguanine adducts in the DNA of cells, characterized by low levels of O^6-alkylguanine-DNA alkyltransferase (OGAT)

activity and a functional mismatch repair (MR) system [5, 6, 12]. In clinical protocols, TZM is administered once daily for five consecutive days, due to the schedule-dependency of the antitumor activity that is essentially related to the cumulative depletion of OGAT activity observed during therapy [14, 19, 24]. The reduction in OGAT activity has been attributed to its consumption in the process of O^6-methylguanine adduct removal.

In MR-deficient leukemic cells, TZM becomes cytotoxic when combined with poly(ADP-ribose) polymerase (PARP) inhibitors [26, 27, 28]. The growth-inhibitory effect induced by the association of TZM and PARP inhibitor is unlikely to be due to O^6-methylguanine since these cells are tolerant of the cytotoxicity mediated by this adduct. Besides low levels of O^6-methylguanine (9%), TZM generates a number of methyl adducts such as N^7-methylguanine (70%) and N^3-methyladenine (10%), that are promptly repaired by the base excision repair (BER) system [19]. The first step in the BER process is the excision of the modified base by damage-specific DNA glycosylases such as methylpurine glycosylase (MPG). The removal of the abasic residue results in the generation of apurinic/apyrimidinic sites which are finally repaired by the coordinated intervention of PARP, DNA polymerase β, X-ray repair cross-complementing 1 (XRCC1), and ligases I and III (reviewed in references 4 and 33). When BER function is inhibited by inactivation of PARP, unrepaired methyl adducts different from O^6-methylguanine might contribute to TZM-induced cytotoxicity.

The aim of the present study was to establish whether different schedules of treatment (using the same total dose of TZM and PARP inhibitors) might affect the overall antitumor activity of the drug combination. In particular, the effects on cell growth and clastogenicity of a single treatment with 125 μ*M* TZM and PARP inhibitor (single exposure) versus two repeated treatments with 62.5 μ*M* TZM and PARP inhibitor (split exposure) were tested. The experiments were performed in MR-deficient and OGAT-proficient Jurkat leukemic cells [15, 25], and TZM was combined with 3-aminobenzamide (AB) or the more potent PARP inhibitor, 8-hydroxy-2-methylquinazolin-4[^{3}H]-one (NU1025) [10]. The results indicated that the split exposure of Jurkat cells induced a more pronounced and prolonged growth inhibition than the single exposure. Moreover, the split exposure downregulated BER transcripts and MPG activity. In addition, PARP inhibitors potentiated the cytotoxic effects induced by TZM in fresh leukemic blasts.

Materials and methods

Cell line and culture conditions

The T-lymphoblastic leukemia cell line Jurkat was purchased from ATCC and cultured in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco), 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, McLean, Va.), at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of leukemic blasts

Leukemic cells (Leu) were obtained from the peripheral blood of patients with acute leukemias. The nonlymphoid acute leukemias were classified (according to the French American British classification) as follows: Leu1, M5A; Leu2, M2; Leu4, M2. The acute lymphoid leukemia was of B origin (Leu3). Blasts were isolated using a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). All samples contained > 80% leukemic blasts, as assessed by Giemsa staining.

Drugs

TZM was kindly provided by Schering-Plough Research Institute (Kenilworth, N.J.). AB was purchased from Sigma (St. Louis, Mo.) and NU1025 was kindly provided by R.J. Griffin (Department of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, UK). Drug stock solutions were prepared by dissolving TZM (100 m*M*) or NU1025 (25 m*M*) in dimethyl sulfoxide, and AB in RPMI-1640 (16 m*M*). The final concentration of dimethyl sulfoxide in drug-treated cultures was always less than 0.5% (v/v) and did not contribute to toxicity (data not shown). Inhibition of PARP was obtained by treating the cells with 2 or 8 m*M* AB or 25 or 50 μ*M* NU1025, concentrations that are known to abrogate PARP activity [1]. TZM was added to cell cultures immediately after AB or NU1025 at concentrations of 62.5 μ*M* or 125 μ*M*.

Drug treatment and cell growth evaluation

Cells were cultured in flasks (Falcon, Becton Dickinson Labware, Oxnard, Calif.) at 3×10^5 cells/ml. Treatments were as follows: in single treatments (1× treatment), cells were exposed once to 62.5 or 125 μ*M* TZM alone or combined with PARP inhibitors (4 or 8 m*M* AB, 25 or 50 μ*M* NU1025) on day 0; in double treatments (2× treatment), cells were exposed to 62.5 μ*M* TZM alone or combined with PARP inhibitors (4 m*M* AB, 25 μ*M* NU1025) on day 0 and again on day 1.

The single treatment with 125 μ*M* TZM plus PARP inhibitor (8 m*M* AB or 50 μ*M* NU1025) is referred to as a single exposure, and the double treatment with 62.5 μ*M* TZM plus PARP inhibitor (4 m*M* AB or 25 μ*M* NU1025) is referred to as a split exposure.

Cells were then incubated at 37°C for an additional 3 days (Jurkat cells) or 1 day (leukemic blasts). Cell growth was evaluated every 24 h by counting viable cells in quadruplicate. Cell viability was determined by trypan blue dye exclusion.

Flow cytometric analysis

Untreated or treated Jurkat cells were harvested after 4 days of culture and fixed with 70% ethanol at –20°C overnight. Cells were washed and resuspended in a staining solution containing 0.1% sodium citrate, RNAse (10 μg/ml), propidium iodide (50 μg/ml) and 0.1% Triton-X, and incubated at 37°C for 10 min. The DNA content of 10^4 cells was determined by flow cytometry (FACScan, Becton Dickinson, San Jose, Calif.). Data collection was gated utilizing forward light scatter and side light scatter to exclude cell debris and aggregates. Apoptotic cells were represented by a broad hypodiploid peak, which was easily distinguishable from the narrow peak of cells with a diploid DNA content in the red fluorescence channel.

Cell survival assay (colony-forming ability)

Cell survival was also assessed by a colony formation assay in the culture medium [35]. Untreated or drug-treated Jurkat cells were seeded at ten cells per well in 96-well plates (round-bottomed) and cultured. After 2 weeks colonies were counted to determine cloning efficiency.

Analysis of chromosome aberrations

For chromosomal analysis, cell cultures were incubated for 2 h with 0.8 μg/ml colchicine (Sigma) before harvesting. Metaphase preparations were obtained according to standard techniques, treating cells first with a hypotonic solution (0.075 *M* KCl) and then with a 3:1 methanol/acetic acid fixative mixture. Air-dried slides were stained with 5% Giemsa in phosphate buffer.

Chromatid and chromosome breaks were evaluated by analyzing 100 metaphases for each culture. Chromatid exchanges (tri- and tetra-radials) were counted as two chromatid breaks and chromosome exchanges (dicentric and translocations) as two chromosome breaks. Statistical analysis was performed using the normal standard deviation as described previously [31].

Northern blot analysis

Total cellular RNA was extracted using the TriPure isolation reagent (Roche, Milan, Italy). RNAs (15 μg) were fractionated by electrophoresis on a formaldehyde-containing 1.2% agarose gel. The integrity of RNA was confirmed by RNA visualization, adding ethidium bromide to the RNA gel loading buffer. RNA was transferred to a nylon membrane (Gene Screen Plus, NEN Life Science Products, Mass.) and hybridized at 68°C for 1 h with a [^{32}P]-labeled probe, using QuickHyb hybridization solution (Stratagene, Cambridge, UK). The blots were washed according to the manufacturer's instructions. The blotted membrane was exposed to X-ray film at –80°C.

The DNA probes used for the detection of XRCC1, MPG and PARP transcripts were obtained as previously described [2, 23, 28]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, a 0.9 kb Eco RI fragment of the human GAPDH gene, was a generous gift from Dr. R. Dalla Favera (Department of Pathology, Columbia University, New York, N.Y.). Bidimensional densitometry of the blots was performed using a scanning imaging densitometer apparatus (GS-670; BioRad, Richmond, Calif.).

Measurement of MPG activity

MPG activity was assayed as previously described [30]. Tumor cells (10^7) were sonicated at 4°C in 0.5 ml buffer I (50 m*M* Tris-HCl, 3 m*M* dithiothreitol, and 2 m*M* EDTA, pH 8.3), with freshly added 1 m*M* 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride. After removal of cell debris by centrifugation, supernatants were immediately tested for MPG activity. Various amounts of tumor cell extracts were incubated with 10 μg (10,000 cpm) freshly dissolved calf thymus DNA methylated by *N*-[^{3}H]-methyl-*N*-nitrosourea (19 Ci/mmol, Amersham) in a total volume of 100 μl buffer II (20 m*M* Tris-HCl, 1 m*M* dithiothreitol, 60 m*M* NaCl, and 1 m*M* EDTA, pH 8). After 1 h at 37°C, the reaction was stopped on ice by the addition of 30 μl 2 *M* NaCl containing 0.5 mg/ml calf thymus DNA and 1 mg/ml bovine serum albumin. DNA was ethanol-precipitated and samples were centrifuged at 10,000 *g* for 15 min. A 300-μl aliquot of supernatant was transferred to a scintillation tube and counted. The MPG activity was determined for protein and time-limiting conditions and is expressed as femtomoles methyl purines released per milligram protein per hour.

Results

Cytotoxic and clastogenic effects of split exposure of MR-deficient leukemic cells to TZM combined with PARP inhibitors

Previous studies have indicated that a peak plasma concentration of 12.1 μg/ml TZM (i.e. 62.5 μ*M*) can be reached when fractionated doses of 200 mg/m^2 are given daily [7]. Based on this observation, we tested the cytotoxic effects of two exposures of tumor cells to 62.5 μ*M* TZM combined with PARP inhibitor (4 m*M* AB or 25 μ*M* NU1025) with an interval of 24 h between exposures (i.e. the split exposure). In this regard, it should be pointed out that since the half-life of TZM in aqueous solution is approximately 90 min, it can be assumed that no residual active drug was present 24 h after treatment. Moreover, the PARP inhibitor concentration used was selected on the basis of experiments with graded concentrations of AB (2 or 8 m*M*) or NU1025 (12.5 or 50 μ*M*) which indicated that a maximal potentiation of TZM cytotoxicity could be achieved with 4 m*M* AB and 25 μ*M* NU1025 (data not shown).

The results obtained from the split exposure were compared with those obtained following the single exposure, i.e. adding 125 μ*M* TZM and PARP inhibitors (8 m*M* AB or 50 μ*M* NU1025) to the cell cultures on day 0. Cell growth was evaluated at daily intervals during 4 days of culture. The results, illustrated in Fig. 1, indicated that: (1) the split exposure induced growth arrest and cells did not recover from the inhibitory effect; (2) a transient growth inhibition was observed when the total dose of TZM or of the PARP inhibitors was given as a single exposure or when the combination of 62.5 μM TZM and PARP inhibitor was used once; (3) exposure of Jurkat cells to 125 μ*M* TZM induced only a slight reduction in the growth rate (similar results were obtained when this treatment was split; data not shown); (4) cells exposed to AB or NU1025 showed a pattern of growth similar to that of untreated controls.

The effect of the split exposure on long-term survival was assessed by a colony-formation assay. The results, illustrated in Fig. 2A, showed that this treatment significantly reduced the ability of Jurkat cells to form colonies, with respect to controls (fourfold reduction) or to cells exposed to the single combined treatment (about a threefold reduction). None of the other double treatments significantly affected colony forming ability.

The split exposure also induced more pronounced apoptotic effects than any other treatment (Fig. 2B). In particular, apoptosis was about twofold higher than that provoked by the single combined treatment. Flow cytometry profiles derived from a representative experiment are illustrated in Fig. 3.

The clastogenic effects of graded TZM concentrations (62.5, 125 and 250 μ*M*), alone or combined with AB (4 m*M*), were evaluated on day 1 (Fig. 4A). TZM was devoid of substantial clastogenicity, even when used at high concentrations. AB significantly enhanced the clastogenic effects of 125 μ*M* ($P < 0.05$) and 250 μ*M* ($P < 0.01$) TZM. In contrast, with 62.5 μ*M* TZM, the slight increase in chromosome aberrations induced by AB was not statistically significant. Therefore, 62.5 μ*M* TZM and PARP inhibitor were added again on day 1 (i.e. split exposure) to cell cultures and chromosome damage was evaluated on day 2. The results illustrated in Fig. 4B, show that the split exposure significantly

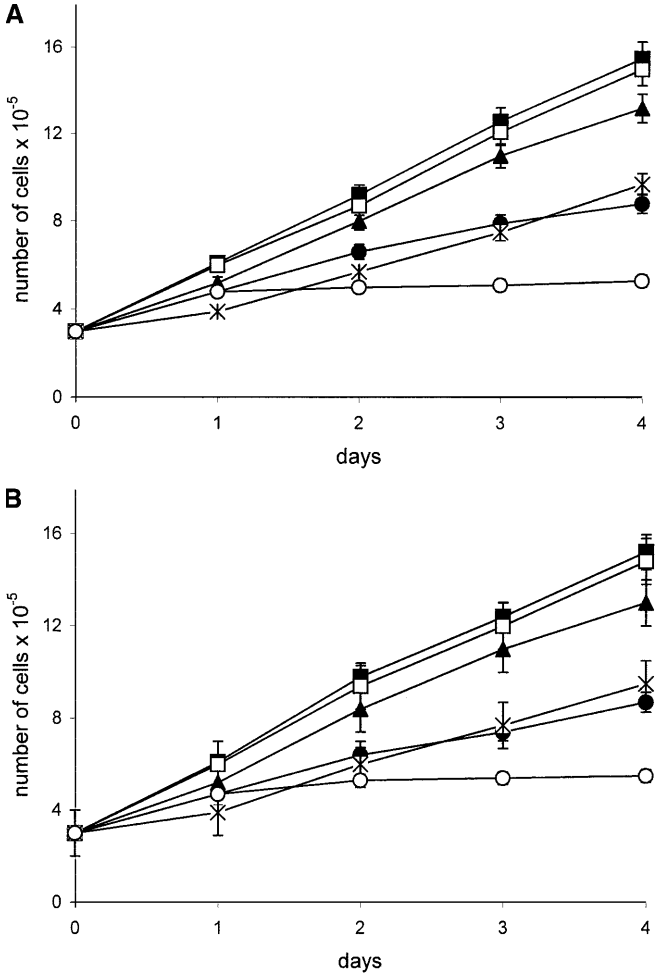

Fig. 1A, B Effects on growth of leukemic cells of split versus single exposure to TZM combined with PARP inhibitors. Jurkat cells were treated with TZM and/or PARP inhibitors AB (**A**) or NU1025 (**B**). Cell growth was evaluated in terms of number of viable cells. **A** ■ untreated cells, ❑ single treatment 8 m*M* AB, ▲ single treatment 125 μ*M* TZM, single treatment 8 m*M* AB + 125 μ*M* TZM (i.e. single exposure), ● single treatment 4 m*M* AB + 62.5 μ*M* TZM, ❍ double treatment 4 m*M* AB + 62.5 μ*M* TZM (i.e. split exposure). **B** ■ untreated cells, ❑ single treatment 50 μ*M* NU1025, △ single treatment 125 μ*M* TZM, ✱ single treatment 50 μ*M* NU1025 + 125 μ*M* TZM (i.e. single exposure), ● single treatment 25 μ*M* NU1025 + 62.5 μ*M* TZM, ❍ double treatment 25 μ*M* NU1025 + 62.5 μ*M* TZM (i.e. split exposure). On days 3 and 4 regression analysis applied to the growth inhibition of cells exposed to the split exposure showed statistically significant differences ($P < 0.01$) with respect to all other groups. Each symbol value represents the mean of cell counts performed in quadruplicate (*bars* ± SE). The results are from one representative of three repeated experiments

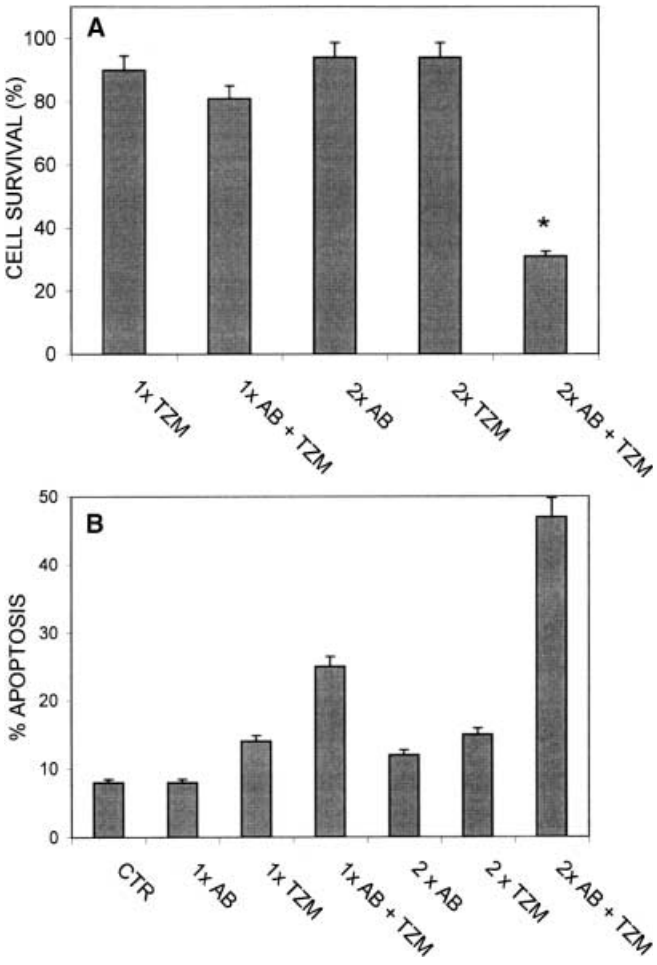

Fig. 2A, B Effects of split exposure of Jurkat cells on colony-forming ability and apoptosis. Jurkat cells were treated as described in the Materials and methods section. The drug concentrations used were 4 m*M* AB and 62.5 μ*M* TZM (*1*× single treatment, *2*× double treatment). **A** Untreated or drug-treated cells were seeded in 96-well plates. After 14 days colonies were counted. The columns indicate the colonies formed by drug-treated cells expressed as percentages of the colonies formed by untreated controls. The indicated means and the relative standard errors (*bars*) were calculated following angular transformation of the percentages. The values are the mean of two independent experiments (two plates per treatment group in each experiment) (*$P < 0.01$). **B** Apoptosis was assessed by flow cytometric analysis of the sub-G_1 DNA content on day 3. The results are expressed as the percentages of apoptotic cells and the values are the means (+SE) of three independent experiments calculated following angular transformation of the percentage values

increased the number of chromosome aberrations in contrast to the effects of the other treatments.

Cytotoxic effects of split exposure in freshly isolated leukemic blasts

Freshly isolated leukemic blasts derived from patients affected by acute leukemia were exposed to 62.5 μ*M* TZM alone or combined with 4 m*M* AB. On day 1, TZM and/or PARP inhibitor were added again to the cultures and the cell numbers were determined on day 2 after seeding. Figure 5 illustrates the results obtained with blasts derived from four different patients. In all cases the split exposure induced a more pronounced reduction in cell numbers than any other treatment. Similar results were obtained when 25 μ*M* NU1025 was used as PARP inhibitor (Fig. 5B). In all cultures TZM was devoid of substantial cytotoxic effects (Fig. 5).

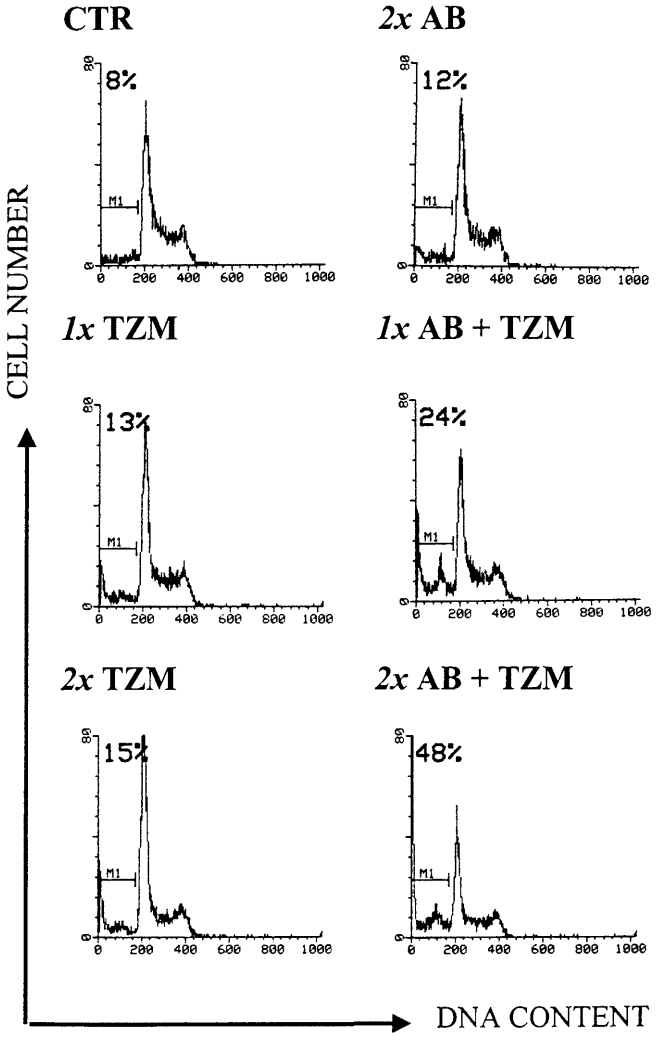

Fig. 3 Flow cytometric analysis of apoptosis in Jurkat cells exposed to 62.5 μ*M* TZM combined with PARP inhibitor (4 m*M* AB). The percentages of cells exhibiting the hypodiploid peak typical of apoptosis are indicated. The percentage of apoptotic cells in the group exposed to a single treatment with AB was 8% (*1x* single treatment, *2x* double treatment)

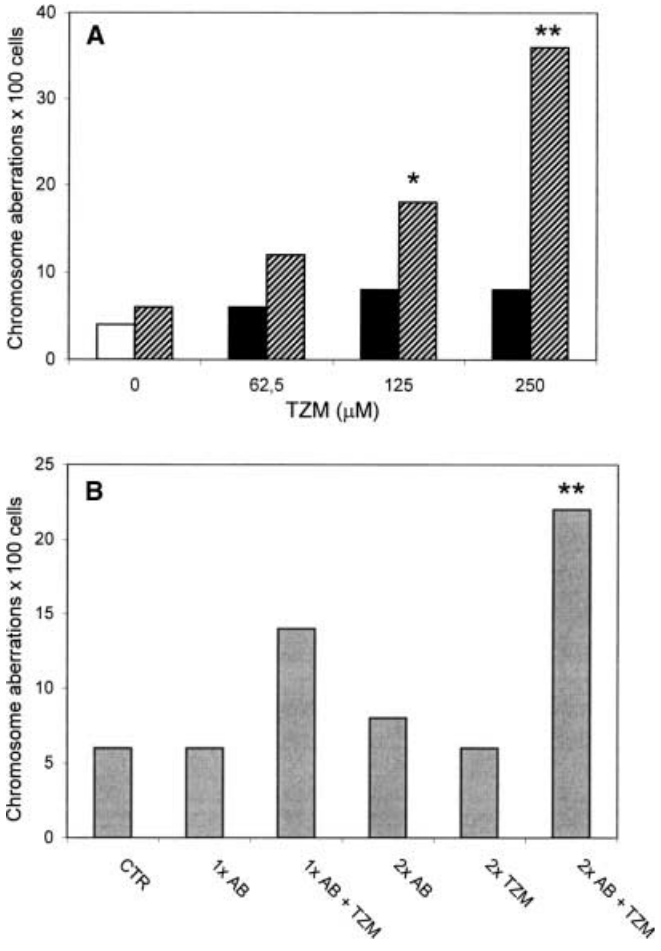

Fig. 4A, B Chromosome aberrations induced by treatment of Jurkat cells with TZM alone or in combination with AB. **A** Jurkat cells were exposed to the indicated concentrations of TZM alone or combined with AB, and chromosome aberrations were analyzed on day 1 (*open bar* untreated cells, *solid bars* TZM-treated groups, *hatched bars* AB or AB + TZM-treated groups; $^{**}P < 0.01$, $^{*}P < 0.05$). **B** Chromosome aberrations were analyzed in Jurkat cells exposed to the split exposure treatment (double treatment with 4 m*M* AB and 62.5 μ*M* TZM). Cells were harvested on day 2 and analyzed for chromosome aberrations. Untreated controls, single and double treatments with AB, single and double treatments with TZM, and single treatment with AB + TZM are also shown ($^{**}P < 0.01$ vs all other treatments; *1x* single treatment, *2x* double treatment)

Effects of split exposure on transcription of BER components and MPG activity

Experiments were carried out to explore whether the expression of BER components might be affected by split exposure to clinical concentrations of TZM combined with AB. Jurkat cells were exposed to single or double treatments with 62.5 μ*M* TZM and/or 4 m*M* AB and tested on day 2 after seeding for the expression of BER gene transcripts by Northern blot analysis. The results of a representative experiment, carried out using PARP, XRCC1 or MPG probes, are illustrated in Fig. 6. The levels of gene transcripts were quantified by densitometric scanning of the autoradiograms and normalized in relation to GAPDH expression. The optical density ratios between treated and control groups are shown in Fig. 6. A reduction in XRCC1 and MPG transcripts occurred in the groups that received a double treatment with TZM. The decrease was particularly evident in cells receiving the split exposure and only in this case a statistically significant reduction in MPG activity was observed (Table 1).

Discussion

A functional defect in the MR system represents an important mechanism of tumor cell resistance to methylating agents, including TZM. This has been attributed essentially to the lack of MR-dependent activation of apoptotic pathways in the presence of

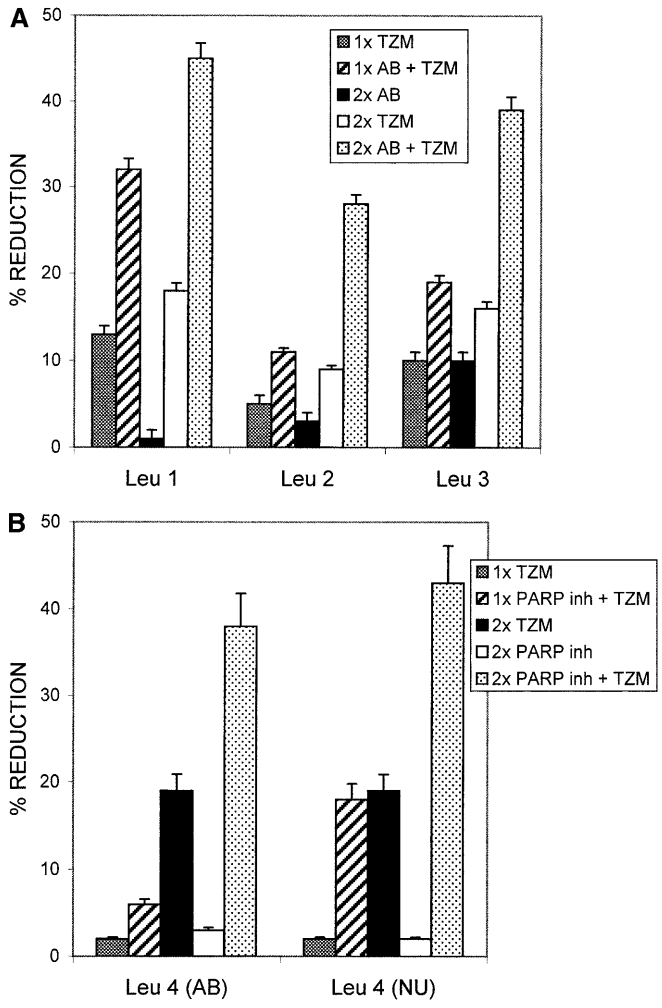

Fig. 5A, B Cytotoxic effects induced by split exposure of freshly isolated leukemic blasts. **A** Freshly isolated blasts from four different patients affected by acute nonlymphoid leukemia (Leu1, Leu2) and acute lymphoid leukemia (Leu3) were treated with 62.5 μ*M* TZM alone or combined with 4 m*M* AB. **B** Freshly isolated blasts from an acute nonlymphoid leukemia patient (Leu4) were treated with 62.5 μ*M* TZM alone or combined with 4 m*M* AB or 25 μ*M* NU1025. In selected groups (indicated as *2x*) treatment was repeated on day 1. Cell numbers were evaluated on day 2. Histograms represent the mean (+SE) percentage cell number reduction in drug-treated samples with respect to the untreated control calculated following angular transformation of the percentage inhibition values determined from quadruplicate counts (*PARP inh* PARP inhibitor AB or NU1025, *1x* single treatment, *2x* double treatment)

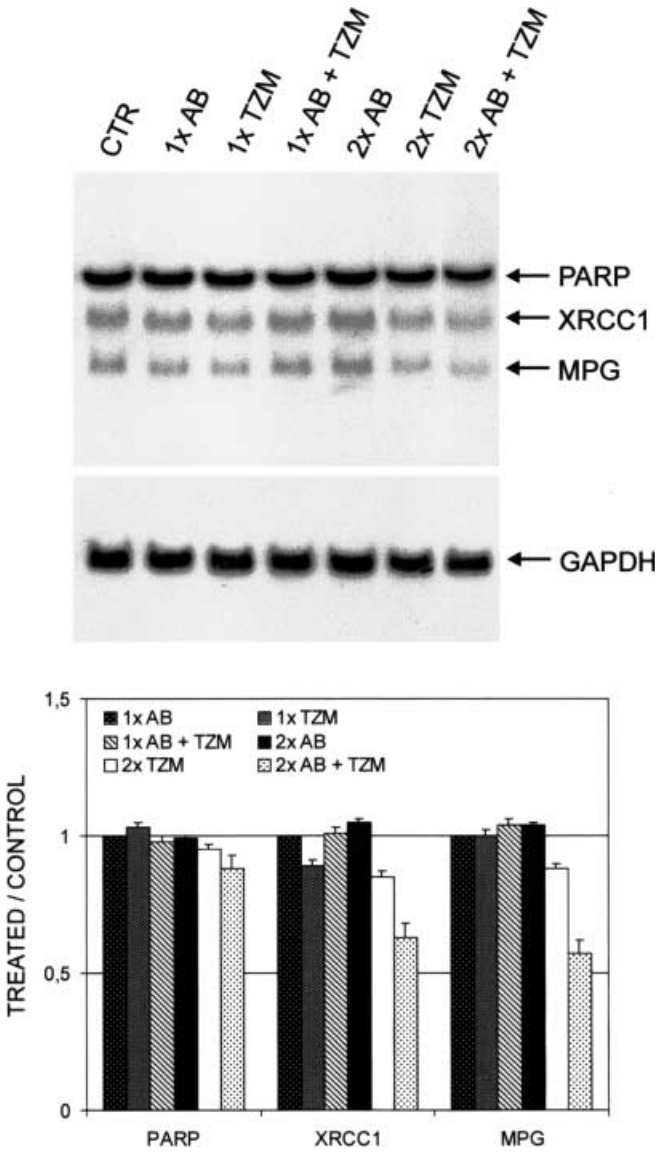

Fig. 6 Expression of PARP, XRCC1 and MPG gene transcripts in Jurkat cells treated with TZM alone or in combination with PARP inhibitor. Jurkat cells were exposed to 62.5 μ*M* TZM and/or 4 m*M* AB and tested on day 2 after seeding for the expression of PARP, XRCC1 and MPG by Northern blot analysis. *Arrows* indicate the XRCC1 (2.1 kb), PARP (3.6 kb) and MPG (1.5 kb) transcripts, respectively. The results are from one representative of two different experiments. The hybridization signals were quantified by densitometric scanning of the autoradiograms and normalized in relation to GAPDH expression (1.2 kb). Histograms indicate the value of the ratio between the optical densities of drug-treated groups and those of controls (untreated cells or cells treated once with AB). Each value represents the mean ratios calculated from two different experiments (*bars* standard deviation)

O^6-methylguanine adducts generated by methylating compounds [6, 12]. However, a therapeutic strategy based on the inhibition of repair of methyl adducts other than O^6-methylguanine might allow the use of these agents in tumor cells with MR deficiency. In fact, downregulation of BER activity by means of PARP inhibitor renders these cells susceptible to cytotoxicity induced by TZM [16, 26, 27, 28].

In the present study we demonstrated that, when the dose of TZM and PARP inhibitor is fractionated, a more pronounced growth inhibitory effect occurs with respect to that induced by an equivalent dose given as a single treatment. The damage produced by split treatment is accompanied by induction of apoptosis, chromosome aberrations and a reduction in colony-forming ability in treated cultures. These results indicate that fractionated doses of TZM and PARP inhibitors would presumably induce extensive DNA damage that cannot

Table 1 Effects of split exposure of Jurkat cells to TZM and AB on MPG activity. Jurkat cells were exposed to 62.5 μ*M* TZM and/or 4 m*M* AB on day 0 (single treatment) or in selected groups treatment was repeated on day 1 after seeding (double treatment). On day 2 cells were harvested and assayed for MPG activity as described in the Material and methods section. The MPG values are the means of four determinations performed using different sample dilutions of the same cell extract. The data are from one representative of two different experiments with comparable results (*CTR* untreated, *1x* single treatment, *2x* double treatment, *SE* standard error, *NS* not significant)

Treatment	MPG (fmol/mg/h[a])	SE	Statistical analysis[b]
CTR	849	32	
1x AB	848	69	NS
1x AB + TZM	964	71	NS
1x TZM	903	167	NS
2x AB	764	54	NS
2x TZM	681	47	NS
2x AB + TZM	563	61	$P < 0.05$

[a] Femtomoles of methyl purines released per milligram of protein per hour
[b] Analysis of the differences between untreated and drug-treated groups using Student's *t*-test

be efficiently repaired. These results appear to be of particular interest taking into account that functional defects in MR have been described in leukemias and lymphomas [11, 36] and linked to the development of lymphoid tumors [13, 21].

The clastogenic properties of TZM have been so far been shown essentially in OGAT-deficient tumors, or in OGAT-proficient cells pretreated with the OGAT inactivator O^6-benzylguanine [3]. In the present study, we demonstrated that in MR-deficient Jurkat cells, that are also endowed with high levels of OGAT activity, interruption of the repair of DNA adducts different from O^6-methylguanine might contribute to the clastogenic properties of TZM. In addition, clastogenic effects derived from inhibition of PARP-mediated repair become evident only when the levels of methylation are sufficiently elevated. In fact, a statistically significant increase in the number of chromosome aberrations was observed when the PARP inhibitor was combined with high TZM concentrations, but the concentrations used are not clinically relevant (125 and 250 μ*M*). Interestingly, when the TZM dose of 125 μ*M* was fractionated into two sequential treatments with 62.5 μ*M* corresponding to the peak plasma concentration reached in patients, chromosome damage was comparable to that induced by the same dose given as a single treatment.

Interest in the clinical potential of TZM for the treatment of hematological malignancies has been raised by recent studies [8, 22]. We report here for the first time that the drug combination of TZM and PARP inhibitor induces cytotoxic effects in freshly isolated leukemic blasts. Moreover, the split exposure with the drug combination showed a net benefit with respect to TZM alone. These results are of particular relevance since the drug combination has been shown to be effective irrespective of the levels of OGAT activity or of the functional status of MR [16, 27, 28].

Previous studies aimed at increasing the antitumor efficacy of methylating agents in tumor cells with an intact MR function by means of the specific OGAT inactivator O^6-benzylguanine have shown that this agent has little effect on the antitumor activity of a single high dose of TZM [32]. In contrast, when the same dose is divided into fractions and given with O^6-benzylguanine, TZM-induced cytotoxicity increases linearly with the dose [32]. This has been attributed to a more pronounced OGAT depletion and elevated O^6-methylguanine damage generated by fractionated treatment with respect to that induced by a single equivalent dose. In the present study, we demonstrated that repeated exposure of MR-deficient cells to TZM alone or combined with PARP inhibitor is accompanied by reduced transcription of BER genes, such as XRCC1 and MPG. In particular, the split exposure significantly decreased MPG activity. Furthermore, we have previously demonstrated that a single exposure of Jurkat cells to high concentrations of TZM (125–500 μ*M*) and AB is followed by increases in XRCC1, MPG and PARP transcripts [28]. Moreover, increased transcription of the XRCC1 transcript also occurs when MR-deficient cells are treated with a methylating agent that almost exclusively induces N^3-methyladenine [29]. It is likely that repeated exposures to TZM would help to keep down resynthesis and delay recovery of BER components.

In conclusion, the results of the present study indicate that the combination of TZM and PARP inhibitor divided into two doses induces more pronounced and long-lasting antitumor effects than a single treatment with an equivalent total dose of both compounds. Moreover, reduction of BER transcripts and MPG activity might presumably contribute to the damage induced by the methylating agent that when combined with PARP inhibitor can also be cytotoxic in the absence of functional MR.

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