O\textsuperscript{6}-Benzylguanine Enhances the In Vitro Immunotoxic Activity of Temozolomide on Natural or Antigen-Dependent Immunity\textsuperscript{1}

ESTER ALVINO, RITA PEPPONI, ELENA PAGANI, PEDRO MIGUEL LACAL, CORRADO NUNZIATA, ENZO BONMASSAR, and STEFANIA D’ATRI

Institute of Experimental Medicine, National Council of Research, Rome, Italy (E.A.); and Istituto Dermopatico Dell’Immacolata, Rome, Italy (R.P., E.P., P.M.L., C.N., E.B., S.D.)

Accepted for publication August 20, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Temozolomide (TMZ) is a new cytotoxic triazene compound of clinical interest that is able to generate methyl adducts at the O\textsuperscript{6}-guanine of DNA, which can be repaired by O\textsuperscript{6}-alkylguanine-DNA alkyltransferase (OGAT). It was previously found that triazene compounds are highly immunosuppressive in mice. In the present study, we investigate whether TMZ could affect immune functions of human competent cells and whether methylation of O\textsuperscript{6}-guanine could be involved in the immunosuppressive activity of the drug. Mononuclear cells (MNCs) obtained from peripheral blood of healthy donors were tested for OGAT activity and treated with TMZ alone or combined with the OGAT inhibitor O\textsuperscript{6}-benzylguanine. Control or drug-treated MNCs were then assayed for natural killer activity and for the ability to proliferate and to generate cytotoxic effector cells in response to interleukin-2 or allogeneic MT-2 tumor cells. The results show that TMZ inhibited both proliferation and induction of lytic activity in response to interleukin-2 or allogeneic MT-2 cells. Moreover, an inverse correlation was found between the OGAT activity of MNCs and their sensitivity to TMZ. The involvement of O\textsuperscript{6}-guanine methylation in the immunosuppressive effects of TMZ was further confirmed by the finding that O\textsuperscript{6}-benzylguanine increased the activity of the drug. On the other hand, the natural killer activity of MNCs was only moderately affected by TMZ, and no relationship was observed between OGAT levels and sensitivity to the drug. These data suggest that in patients with tumors who are undergoing TMZ treatment, the drug may impair immune responses involving cell proliferation, depending on OGAT levels of MNCs, and that O\textsuperscript{6}-benzylguanine may potentiate this activity.

Temozolomide (TMZ) is a methyldemethylating imidazotetrazinone presently under phase II/III clinical investigation for the treatment of various neoplasias, including non-Hodgkin’s lymphoma, melanoma, and high-grade glioma (Bleehen et al., 1995; Woll et al., 1995; Bower et al., 1997). In physiological situations, TMZ readily decomposes into 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC), the methyldemethylating derivative that is also generated through host metabolism of dacarbazine [DTIC; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; Stevens et al., 1987]. MTIC further decomposes into a methyl-diazonium ion that reacts with nitrogen bases in DNA, mainly at the N\textsuperscript{7} and O\textsuperscript{6} positions of guanine (O\textsuperscript{6}-G), N\textsuperscript{3} position of adenine, and O\textsuperscript{6} position of thymine. Adduct formation at O\textsuperscript{6}-G is considered to be one of the major determinants of TMZ cytotoxicity because, in most cases, tumor cell sensitivity to the drug is inversely correlated with the activity of the DNA repair protein O\textsuperscript{6}-alkylguanine-DNA alkytransferase (OGAT; Tisdale, 1987; D’Atri et al., 1995; Tentori et al., 1995, 1997; Liu et al., 1996).

Human OGAT is a 22-kDa protein that appears to be present in varying amounts in normal tissue (Myrnes et al., 1983; Gerson et al., 1986; Citron et al., 1991). The content of OGAT also varies among individuals (Myrnes et al., 1983; Pegg, 1990; Citron et al., 1991) and is highly heterogeneous among tumor lines (D’Incalci et al., 1988; Fornace et al., 1990, Pegg, 1990). The protein removes methyl and other short alkyl groups from O\textsuperscript{6}-G and transfers them to an internal cysteine residue in a stoichiometric and autoinactivating reaction (reviewed in Pegg, 1990). The extent of O\textsuperscript{6}-alkylguanine repair is therefore dependent on the initial cellular OGAT levels and on the rate at which de novo synthesis of the protein occurs.

ABBREVIATIONS: TMZ, temozolomide (8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one); BG, O\textsuperscript{6}-benzylguanine; CM, complete medium; CTL, cytotoxic T lymphocytes; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; E, effector; O\textsuperscript{6}-G, O\textsuperscript{6}-guanine; \textsuperscript{3}H-TdR, [methyl\textsuperscript{3}]thymine deoxyriboside; IL, interleukin; LAK, lymphokine-activated killer; O\textsuperscript{6}-MeG, O\textsuperscript{6}-methylguanine; MNC, mononuclear cell; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide; NK, natural killer; OGAT, O\textsuperscript{6}-alkylguanine-DNA alkyltransferase; T, target.
Depletion of OGAT in tumors proficient for this protein, is considered to be a useful strategy to increase clinical responses to O6-G-methylating agents (Dolan and Pegg, 1997). Indeed, depletion of OGAT activity by the O6-benzylguanine (BG), which acts as a potent substrate for the protein (Dolan et al., 1990), has been shown to increase tumor cell sensitivity to TMZ both in vitro (Bauer et al., 1993; Tentori et al., 1995, 1997; Liu et al., 1996; Wedge et al., 1996a) and in vivo (Wedge and Newlands, 1996b; Wedge, 1997).

The mechanism by which the persistence of O6-methylguanine (O6-MeG) in DNA leads to cell cytotoxicity involves recognition of O6-MeG-T and O6-MeG-C mispairs, occurring during cell duplication, by the mismatch repair system (reviewed in Jiricny, 1996; Modrich, 1997). Thus, independent of their OGAT activity, tumor cell lines harboring mutations in the constituent proteins of the mismatch repair system are resistant to the cytotoxic effects of TMZ (Liu et al., 1996; D’Atri et al., 1998) and other O6-G methylating agents (Karan and Bignami, 1992; Kat et al., 1993; Carethers et al., 1996).

A number of studies are available suggesting that stimulation of the host’s immune system with immunomodulating agents or antitumor vaccines might be of benefit in the treatment of patients with cancer, particularly in the case of minimal residual disease (Ockert et al., 1999). Immunotherapy protocols based on DTIC plus interferon and/or interleukin (IL)-2 administration are already used in the treatment of metastatic melanoma (Cohen and Falkson, 1998). In these protocols, it is assumed that stimulation of the host’s immune function results in better clinical responses.

Previous studies performed in the murine model have shown that triazene compounds not only possess antitumor activity but also are immunosuppressive. Indeed, humoral and cell-mediated immune responses are profoundly inhibited in mice exposed to DTIC (Giampietri and Bonmassar, 1978; Nardelli et al., 1984). Moreover, murine splenocytes treated in vitro with the drug show a marked impairment of antigen-dependent cell-mediated immunity and of proliferative responses to mitogens (Giampietri and Bonmassar, 1978; Nardelli et al., 1984). On the other hand, the effects of triazene compounds on immune function of human competent cell lines have not been extensively investigated (Bonmassar et al., 1994), and no data are presently available on the immunosuppressive activity of TMZ.

As already occurring for DTIC, it is possible that TMZ could be used in association with immunomodulating agents in the treatment of melanoma. Therefore, studies have been performed to investigate whether the molecular mechanisms underlying the antitumor activity of TMZ may also be responsible for immunosuppressive effects. This was done to obtain useful information for a rational approach to cancer treatment based on TMZ associated with biological response modifiers. The present report illustrates the in vitro effects of TMZ, alone or in combination with BG, on natural or antigen-dependent cell-mediated immunity of human peripheral blood mononuclear cells (MNCs).

**Materials and Methods**

**Cell Lines and Culture Conditions.** Erythroleukemia, K562, and Burkitt lymphoma, Daudi, cell lines were obtained from American Type Culture Collection (Rockville, MD). The human MT-2 cell line human T-cell leukemia virus type I-transformed cord blood T lymphocytes; Miyoshi et al., 1981) was kindly provided by Dr. B. Macchi (University of Tor Vergata, Rome, Italy). This cell line expresses class I and class II HLA antigens and B7 molecule and was used to elicit allogeneic antigen-dependent immune responses in human MNCs.

All cell lines were maintained in RPMI 1640 (HyClone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS; HyClone), 2 mM l-glutamine, and antibiotics (Flow Laboratories, McLean, VA) and cultured at 37°C in 5% CO2 humidified atmosphere.

**Chemical and Biological Agents.** TMZ was kindly provided by Sehering-Florey Research Institute (Kenilworth, NJ). Because the drug readily decomposes in aqueous solution into MTIC (Stevens et al., 1987), solutions were always prepared fresh by dissolving the drug in RPMI 1640. The solutions were protected from light and used immediately after preparation. The OGAT inhibitor BG was synthesized and generously donated by Prof. L. Lassiani (Institute of Pharmacological Chemistry, University of Trieste, Italy). Mitomycin C (Kiowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was diluted in water and stored at ~80°C. Human recombinant IL-2 (kindly provided by Hoffman-La Roche, Basel, Switzerland) was diluted in RPMI 1640 medium and stored at ~20°C.

**Preparation of MNCs and In Vitro Treatment with TMZ and BG.** MNCs were separated from the peripheral blood of healthy donors on a Ficoll-Hypaque gradient and washed twice in PBS. MNCs were then incubated (at 37°C for 1 h) in 50-ml tubes (Falcon; Becton and Dickinson Labware, Franklin Lakes, NJ; 2 × 106 cells/ml, 10 ml/tube) in RPMI 1640 supplemented with 2% FCS, 2 mM l-glutamine, and antibiotics (hereafter referred to as complete medium (CM)) or in CM containing BG at the final concentration of 0.5 μM. Suspensions of control or BG-treated MNCs were then admixed with equal volumes of CM alone or CM containing TMZ to give final drug concentrations ranging between 62.5 and 500 μM and incubated at 37°C for 4 h. Because MNCs were not washed before the addition of TMZ, BG was always present during the 4-h treatment with the triazene compound. Thereafter, MNCs were washed in PBS and tested immediately for cytotoxic activity against K562 target cells (i.e., natural killer (NK) activity). MNCs were also stimulated with IL-2 or allogeneic MT-2 tumor cells either in CM or in CM containing 2 μM BG. MNC responses to IL-2 or MT-2 cells were evaluated in terms of proliferation and generation of lymphokine-activated killer (LAK) cells or antigen-dependent cytotoxic T lymphocytes (CTL, see below).

To measure the effect of multiple exposures to TMZ, control or BG-treated MNCs (1 × 106 cells/ml) were dispensed in 6-well (3 ml/well) or 96-well (0.2 ml/well) plates (Falcon) and treated daily with 50 μM TMZ for 5 days. The 5-day treatment of MNCs preexposed to BG was performed in the presence of BG 2 μM. Four hours after the last TMZ treatment, MNCs were tested for NK activity or stimulated with IL-2 or with allogeneic MT-2 tumor cells. Stimulation of MNCs treated with BG or TMZ plus BG was performed in the presence of BG 2 μM.

**Evaluation of MNC Proliferative Responses.** Control or drug-treated MNCs (1 × 106 cells/ml in CM) were plated onto 96-well microtiter plates (0.2 ml/well) and stimulated with IL-2 (500 IU/ml) or with mitomycin-inactivated (80 μg/ml for 1 h) allogeneic MT-2 tumor cells (5 × 103 cells/well) for 4 or 5 days, respectively. Stimulation of MNCs that had been preexposed to BG or TMZ plus BG was performed either in CM alone or in CM containing BG 2 μM. [methyl-3H]Thymidine deoxyriboside (3H-TdR; Amersham International Plc, Amersham, UK) was added to the cultures (5 μCi/ml) 18 h before the end of incubation, and radioactive incorporation was determined by conventional liquid scintillation counting with a TRI-CARB 1900 counter (Packard Instrument Co., Meriden, CT).

**In Vitro Generation of LAK Cells and CTL.** To generate LAK cells, control or drug-treated MNCs (1 × 106/ml in CM) were dis-
pense (3 ml/well) in 6-well plates and incubated with 500 IU/ml IL-2 at 37°C for 4 days. To generate allosensitized CTL, MNCs were plated as above and cocultured with mitomycin-inactivated MT-2 tumor cells (7.5 × 10⁶/well) at 37°C for 5 days. Stimulation of MNCs pretreated with BG or TMZ plus BG was performed either in the absence or the presence of BG 2 μM. At the end of incubation period, effector cells were recovered from the cultures and tested for cytotoxic activity against Daudi (LAK cells) or against MT-2 (CTL) target cells with the use of a conventional ¹ⁱ¹In-release assay (see below).

**Cytotoxicity Assay.** Aliquots of K562, Daudi, or MT-2 cells were centrifuged, resuspended in 0.1 ml of FCS, and incubated with 100 μCi of Na₂¹⁵¹CrO₄ (Amersham) at 37°C for 1 h. After incubation, the cells were extensively wash in RPMI 1640 medium, suspended in RPMI 1640 containing 10% FCS and 2 mM l-glutamine, and used in the cytotoxicity assay.

The cytotoxic activity of MNCs was determined using a ⁵¹Cr-release assay (Bonmassar et al., 1994). Effector cells, suspended in RPMI 1640 containing 10% FCS and glutamine, were plated (0.1 ml/well, in quadruplicate) onto U-bottomed 96-microtiter plates (Greiner GmbH, Frickenhausen, Germany) by making serial 2-fold dilutions, starting at a concentration of 2 × 10⁵ cells/ml. ⁵¹Cr-labeled target cells (2 × 10⁴) were then added in a volume of 0.1 ml to give a final volume of 0.2 ml and an effector (E)-to-target (T) cell ratio ranging from 100:1 to 12.5:1. The plates were then centrifuged at 80g for 5 min and incubated at 37°C for 4 h in a 5% CO₂ humidified atmosphere. After incubation, the plates were centrifuged at 250g for 10 min, and 0.1 ml of supernatant was harvested from each well and counted in a gamma-scintillation counter (Cobra II Monodetector, model 5093; Packard Instrument Co.).

**Evaluation of Cytotoxic Activity of Effector Cells.** The percentage of specific target cell lysis was calculated as follows:

\[
\text{Specific lysis (\%)} = \frac{\text{Test cpm} - \text{autologous cpm}}{\text{Total cpm} \times 0.5} \times 100
\]

where test cpm is the mean cpm relative to ⁵¹Cr released in presence of effector cells, autologous cpm is the mean cpm relative to ⁵¹Cr released by target cells incubated with unlabeled autologous cells in place of effector cells, and total cpm is the mean cpm of 2 × 10⁵ ⁵¹Cr-labeled target cells (i.e., total radioactivity incorporated by target cells).

To express cytolytic effects using a single value instead of a concentration-effect curve, cytotoxic activity of effector cells was expressed in terms of the number of target cells lysed (i.e., “sacrificed”) by a fixed number of effector cells, calculated as follows:

\[
KC(m) = \frac{m 	imes T \times n}{E \times n \times 100}
\]

where KC(m) is the number of target cells sacrificed by m effector cells, T is the total number of target cells present in each well, En is the number of effector cells present in each well at the selected E/T cell ratio, and n is the specific lysis produced theoretically by En effector cells. The n% value is extrapolated from the best-fit curve obtained by plotting the different percentages of specific lysis against the logarithm of the number of effector cells/well. In the present study, KC(m) was calculated for m = 1 × 10⁶ cells (i.e., KC(10⁶)), and En = 2 × 10⁵ cells (i.e., at an E/T cell ratio of 100:1).

Cytotoxic activity of LAK cells and CTL was also expressed in terms of KC(culture) (i.e., the number of target cells potentially lysed by the total number of effector cells present in each culture at the end of stimulation period (calculated multiplying KC(10⁶) values by the total number of effector cells recovered from each culture)). This parameter was introduced to illustrate the effect of TMZ on activation and expansion of cytotoxic effector cells using a single value.

**Evaluation of MNC Sensitivity to TMZ.** MNC sensitivity to TMZ was expressed in terms of the 50% inhibitory drug concentration (IC₅₀, or the concentration of drug capable of inhibiting MNC proliferative responses or cytotoxic activity by 50%) calculated on the regression line in which cpm (for proliferative responses) or KC(10⁶) or KC(culture) for cytotoxic activity values were plotted against the logarithm of drug concentration.

In selected cases, IC₅₀ data were accompanied by the respective confidential intervals calculated on the bases of a p value of .05.

**OGAT Assay.** MNCs, either untreated or exposed to BG, were washed twice with PBS and stored as pellets at −80°C until used. OGAT activity was determined by measuring the transfer of ³H-methyl groups from a DNA substrate to the OGAT protein (Morten and Margison, 1988). Briefly, cell pellets were thawed, resuspended in 1 ml of lysis buffer (0.5% 3-[3-cholamidopropyl]dimethylammonio)propanesulfonate, 50 mM Tris · HCl pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 2 mg/ml leupeptine), and incubated for 30 min at 4°C. Cell lysates were then centrifuged at 15,000 rpm for 10 min. Aliquots of supernatants were then diluted in 50 mM Tris · HCl buffer, pH 8.3, containing 1 mM EDTA and 3 mM dithiothreitol and incubated with 10 μg of ³H-methylated DNA at! 37°C for 1 h. DNA was then hydrolyzed by heating samples at 75°C for 45 min in the presence of 1 N perchloric acid and protein precipitated with 1 mg of BSA as carrier. Pellets were washed with 1 N perchloric acid and resuspended in 0.01 N NaOH, and radioactivity counted in a liquid scintillation counter (TRI-CARB 1900) after the addition of scintillation liquid (Ultima Gold; Packard Instruments, Groningen, the Netherlands). Protein concentration in supernatants was evaluated according to the method of Bradford (1976) using the Bio-Rad (Hercules, CA) Dye Solution and BSA as standard. OGAT activity was expressed in terms of fmol ³H-methyl groups transferred/mg of protein in cell extract.

**Statistical Analysis.** Differences in proliferative responses of control or drug-treated MNCs were evaluated with the use of the Student’s t test. Differences in cytotoxic activity of control or drug-treated MNCs were evaluated taking into account the percentage of specific cytoxicity at all E/T ratios; therefore, p values were calculated using ANCOVA performed on the regression of the percentage of specific ⁵¹Cr release over the logarithm of the number of effector cells/well. All the data relative to cell-mediated cytolysis were expressed in terms of KC(10⁶) or KC(culture) values, without conventional S.E. or S.D. values. Actually, no statistical analysis can be performed using these parameters, which are not suitable for ANCOVA of regression lines.

**Results**

**OGAT Activity of MNCs Obtained from Peripheral Blood of Healthy Donors.** The level of OGAT activity was evaluated in MNCs obtained from 22 different healthy donors. The results (Fig. 1) show that a wide range of enzyme activity (370–1500 fmol/mg protein) was detectable in MNCs. However, the majority of the samples showed OGAT levels ranging from 800 to 1100 fmol/mg protein; only 3 of 22 samples showed enzyme activity lower than 500 fmol/mg protein. These MNCs were considered to be endowed with low OGAT levels.

**Relationship between OGAT Activity of MNCs and TMZ-Mediated Impairment of Proliferative Responses Induced by IL-2 or by Allosensitization with MT-2 Cells.** The effects of TMZ on MNC proliferative responses induced by IL-2 or inactivated allogeneic MT-2 tumor cells were evaluated in samples endowed with different OGAT levels. The relationship between OGAT activity and MNC sensitivity to TMZ is illustrated in Fig. 2. The results show that TMZ inhibited MNC proliferation elicited by IL-2 (Fig. 2A) or by allosimulation (Fig. 2B) to comparable extents, with IC₅₀ values ranging between 134 and 515 μM. In both cases, MNC sensitivity to the drug was inversely correlated with OGAT activity.
Relationship between OGAT Activity of MNCs and TMZ-Induced Impairment of NK Function and of LAK Cell and CTL Generation. The effect of TMZ on NK function was evaluated in three MNC samples endowed with markedly different enzyme activity. The results illustrated in Table 1 shows that TMZ inhibited moderately the NK function, with the IC50 of the agent being about 400 μM. However, these inhibitory effects were not clearly dependent on the level of OGAT activity, being similar for all the three MNC preparations used.

The effects of TMZ on LAK cell or CTL generation were evaluated in five MNC samples expressing different OGAT activity. For each sample, IC50 values were calculated on the regression lines obtained by plotting either KC(106) or KC(culture) against the logarithm of drug concentration. Correlation coefficients were 0.92 (F) and 0.98 (E) for A and 0.94 (F) and 0.99 (E) for B.

TABLE 1
Effect of TMZ on NK activity of three MNC samples endowed with different OGAT activity

<table>
<thead>
<tr>
<th>Donor</th>
<th>OGAT Activity</th>
<th>TMZ IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>445 ± 19</td>
<td>361 (285–455)</td>
</tr>
<tr>
<td>10</td>
<td>792 ± 26</td>
<td>362 (316–413)</td>
</tr>
<tr>
<td>9</td>
<td>1400 ± 46</td>
<td>408 (309–539)</td>
</tr>
</tbody>
</table>

a MNC, obtained from the indicated donors, were exposed to graded concentration of TMZ (62.5–500 μM) for 4 h, washed, and tested for cytotoxic activity against K562 target cells, using a 51Cr-release assay (see Materials and Methods).

b OGAT activity is expressed in terms of fmol of 3H-methyl groups transferred per mg of protein in cell extract. Each value represents the arithmetic mean ± S.E of at least three independent experiments.

c MNC sensitivity to TMZ is expressed in terms of IC50, i.e., the concentration of drug capable of inhibiting MNC cytotoxic activity by 50%, calculated on the regression line in which KC(106) values were plotted against the logarithm of drug concentration. In parentheses, confidential limits of IC50.
that the values of KC MNCs. Comparable inhibition of LAK cell and CTL generation impairment of both proliferation and lytic activity induction of MNCs. Comparable inhibition of LAK cell and CTL generation was observed when IC_{50} values were calculated using KC (10^6). On the other hand, TMZ appeared to be slightly more effective in reducing CTL than LAK cell generation when IC_{50} was calculated with KC(culture).

**Influence of BG on TMZ-Induced Impairment of Cell-Mediated Immune Responses of MNCs.** To further confirm the relationship between OGAT activity and MNC sensitivity to TMZ, the influence of BG on TMZ-mediated impairment of immune responses induced by IL-2 or by allogeneic MT-2 cells was investigated. Studies were performed to establish whether exposure to BG before and during TMZ treatment was sufficient to increase MNC sensitivity to the triazene compound. It was also tested whether additional exposure to the OGAT inhibitor after TMZ treatment (i.e., during the stimulation period with IL-2 or MT-2 cells) was required for amplification of the suppressive effects of the triazene compound on MNC function.

The results of Fig. 4 refer to a representative experiment performed with MNCs obtained from donor 21, endowed with OGAT activity of 903 ± 77 fmol/mg protein (i.e., belonging to the most representative class of OGAT values). Cell suspension was treated with TMZ or TMZ plus BG and then tested for proliferative responses after stimulation with IL-2 (Fig. 4A) or with allogeneic MT-2 cells (Fig. 4B). Stimulation of MNCs exposed to BG or TMZ plus BG was performed either in CM or in CM containing 2 μM BG. The results show that: 1) significant impairment of proliferative responses of MNCs treated with TMZ alone occurred only at concentrations of 250 and 500 μM; 2) exposure to BG before, during, and after treatment with TMZ (i.e., “continuous exposure” to BG) substantially reduced the IC_{50} value of the triazene compound (see legend for Fig. 4); 3) continuous exposure to BG alone did not affect MNC proliferative responses and 4) no amplification of the suppressive effect of TMZ was obtained if MNCs pretreated with TMZ plus BG were stimulated without the OGAT inhibitor (see legend for Fig. 4).

The influence of BG on TMZ-mediated immunotoxicity was also investigated in terms of cell-mediated cytolytic effects (Fig. 5), using the same MNCs tested for cell proliferation (i.e., MNCs of donor 21). The results of the experiments performed, according to the same treatment schedules described for Fig. 4, show comparable results for LAK cells (Fig. 5A) and CTL function (Fig. 5B). Actually, the inhibitory effects of TMZ on cytotoxic activity were only amplified by the continuous exposure of MNCs to BG, as demonstrated by IC_{50} values (see legend for Fig. 5).

An evaluation of OGAT activity of MNCs showed that 1-h exposure to BG reduced the enzyme activity to undetectable levels (data not shown). However, complete OGAT recovery occurred within 72 h after BG removal (data not shown). On the other hand, continuous exposure to BG ensured persistent depletion of the enzyme activity (data not shown).

Additional experiments were performed with MNCs obtained from donor 19 (OGAT activity, 375 ± 30 fmol/mg protein), representative of the “low OGAT activity” class. The cells were treated with TMZ or TMZ plus BG and then sensitized with allogeneic MT-2 cells. MNCs pretreated with BG or TMZ plus BG were sensitized either in CM alone or in CM containing 2 μM BG. MNCs proliferation was evaluated by the 3H-TdR incorporation assay and expressed in terms of mean cpm calculated on four replicates. Bars, S.E. The IC_{50} values and their confidential limits for TMZ relative to MNCs proliferative responses to IL-2 (A) were TMZ alone, 216 (196–237) and TMZ plus BG, 149 (118–180). The IC_{50} values and their confidential limits for TMZ relative to MNCs proliferative responses to allogeneic MT-2 cells (B) were TMZ alone, 201 (175–232) and TMZ plus BG, 98 (72–134). Because no overlapping occurred between confidential limits relative to IC_{50}, in all cases differences between IC_{50} values relative to TMZ and IC_{50} values relative to TMZ plus BG were statistically significant (p < .05). When stimulation of MNCs pretreated with TMZ plus BG was performed without the OGAT inhibitor, no increment was observed in the suppressive effect of the triazene compound (data not shown).

**Effect of Multiple TMZ Treatments on Cell-Mediated Immune Function of MNCs.** Pharmacokinetics studies have
shown that patients receiving TMZ on a repeated dose schedule (200 mg/m² × 5 days) attain a maximum plasma concentration of about 50 μM after each TMZ administration (Newlands et al., 1992). We therefore decided to verify whether an in vitro TMZ treatment of MNCs resembling the clinical condition could determine an impairment in cell-mediated immunity.

To this end, MNCs obtained from donor 21 were exposed to multiple TMZ treatments (50 μM/day for 5 days) and then tested for NK activity and for the ability to respond to IL-2 or allogeneic MT2 cells in terms of proliferation and generation of LAK cells or CTL. The effect of BG on MNC sensitivity to TMZ was also evaluated.

The results illustrated in Fig. 7A show that the MNC ability to proliferate in response to IL-2 or MT-2 cells was reduced by TMZ of about 35 to 40%. Moreover, BG treatment resulted in a further and significant (p < .01) increase in TMZ-induced inhibition of proliferative responses. The multiple TMZ treatment also impaired MNC ability to generate...
proliferation-dependent immune functions of human MNCs in vitro. Moreover, for the first time it has been demonstrated that methylation of O\(^6\)-G is, at least in great part, responsible for the immunosuppressive effects of the drug.

Clonal expansion of lymphocytes is central to the induction of both humoral and cellular immune function. It is well documented that a number of antitumor agents exert immunosuppressive activity through a mechanism that is related, at least in part, to the impairment of lymphocyte proliferation induced by several stimuli. The results illustrated in Fig. 2 show that this also occurs in the case of TMZ. The drug markedly inhibited MNC proliferation induced by IL-2 or by allogeneic tumor cells, thus preventing expansion of effector cells. In agreement with previous studies performed on tumor cells (Tisdale, 1987; Baer et al., 1993; D’Atri et al., 1995; Tentori et al., 1995, 1997; Liu et al., 1996; Wedge et al., 1996a), the antiproliferative effects of TMZ were strictly dependent on methylation of \(O^6\)-G because MNCs sensitivity to the drug was inversely correlated with their OGAT activity.

In vitro treatment with TMZ also impaired the generation in cytotoxic effector cells in MNCs incubated with IL-2 (i.e., LAK cells, Fig. 3A) or cocultivated with allogeneic tumor cells (i.e., CTL, Fig. 3B). As observed for proliferative responses induced by the same stimuli, sensitivity to TMZ was inversely correlated with OGAT levels of MNCs. This finding, together with the observation that the IC\(_{50}\) values relative to TMZ calculated on KC(culture) were lower than those calculated on KC(10\(^8\)), suggests that inhibition of LAK cell and CTL generation is largely dependent on the antiproliferative effects of TMZ. This hypothesis is also supported by the observation that NK cell activity, which does not involve MNC expansion, was only moderately affected by TMZ, with no obvious relationship with OGAT function (Table 1). In addition, TMZ-mediated impairment of NK activity was not influenced by MNC preexposure to BG (data not shown).

The involvement of \(O^6\)-G methylation in the immunosuppressive effects of TMZ is further confirmed by the finding that continuous exposure to BG increased the sensitivity to TMZ of MNCs endowed with high OGAT levels. It must be emphasized that continuous exposure to BG ensured persistent abrogation of OGAT activity of MNCs, as extensively described in Results. Both proliferative responses and generation of cytotoxic effectors induced by IL-2 or allogeneic tumor cells were impaired to a higher extent in MNCs treated with TMZ plus BG with respect to MNCs treated with TMZ alone (Figs. 4 and 5). In MNCs possessing low OGAT levels, the effect of BG on sensitivity to TMZ was limited, and the decrease in the IC\(_{50}\) values, although present, did not reach statistical significance (Fig. 6). However, if the influence of BG was analyzed on the basis of each single TMZ concentration, a potentiating effect of the inhibitor could be demonstrated at the lowest drug concentrations (i.e., 62.5 and 125 \(\mu\)M). On the other hand, when 250 or 500 \(\mu\)M of TMZ was used, the drug alone was highly effective in abrogating immune responses, and therefore exposure of MNCs to BG did not result in a further increase in immunosuppression. Similar patterns of differential effects of BG on the susceptibility to triazenes and other \(O^6\)-G alkylating agents have been found in human tumor cells (Dolan et al., 1991; Baer et al., 1993; Wedge et al., 1996a).

Presently, the treatment schedule recommended for TMZ is 200 mg/m\(^2\) \(\times\) 5 days. Pharmacokinetics studies have
shown that patients receiving TMZ with this schedule attain a maximum plasma concentration of about 50 μM after each TMZ administration (Newlands et al., 1992). The results of Figs. 4, 5, and 6 show that a single treatment with 62.5 μM TMZ (concentration comparable to that obtainable in the clinic) did not impair the immune functions of MNCs endowed with either high or low OGAT activity. However, when MNCs with high enzyme levels were exposed to five subsequent treatments with 50 μM TMZ, a significant inhibition was observed of both proliferative response and cytotoxic effector generation induced by IL-2 or allogeneic tumor cells (Fig. 7). Also in this case, the combined treatment of TMZ plus BG was more effective than TMZ alone in inhibiting MNC immune functions.

Taken together, these data demonstrate that TMZ downregulates both natural and antigen-dependent cell-mediated immune functions in vitro and suggest that this could also occur in patients with cancer treated with the agent. Indeed, it has already been shown that in patients with melanoma receiving TMZ (150 mg/m²) on a 5-day schedule, a significant and progressive depletion of OGAT activity occurred in MNCs, an excess of the toxic O6-MeG lesions and, hence, immunodepression might be expected to take place. Complete suppression of OGAT activity of MNCs might occur preferentially in MNCs endowed with low levels of the protein, as already described in patients with melanoma treated with the methylating triazene compound CB10-277 (Sio et al., 1992).

On the basis of the results obtained in vitro, it is reasonable to predict that the NK function should not be substantially depressed by TMZ shortly after treatment in vivo. On the contrary, the ability of MNCs to generate LAK cells and antigen-dependent CTL is expected to be reduced in patients undergoing TMZ treatment. Therefore, the determination of individual OGAT levels in normal lymphocytes could provide valuable information into the susceptibility of the host to TMZ-induced impairment of the immune system.

The use of BG to potentiate the antitumor effects of TMZ could also result in a further increase of TMZ-induced immunodepression. However, a continuous exposure of MNCs to the agent is required to enhance the immunotoxity of TMZ. These findings should be taken into consideration in designing the protocols of immunochemistry. Actually, treatment with triazenes combined with BG could interfere with the immunostimulating activity of cytokines such as IL-2 or active MHC molecules.

Temozolomide and O6-Benzylguanine Immunotoxicity

in recurrent or progressive high-grade glioma. Cancer Chemother Pharmacol 40: 484–488.


Received April 19, 2017 from JCTJ.
Triazene compounds induce apoptosis in O\textsuperscript{6}-alkylguanine-DNA alkyltransferase deficient leukemia cell lines. *Leukemia (Baltimore)* 9:1888–1895.


Wedge SR and Newlands ES (1996b) O\textsuperscript{6}-Benzylguanine enhances the sensitivity of a glioma xenograft with low O\textsuperscript{6}-alkylguanine-DNA alkyltransferase activity to temozolomide and BCNU. *Br J Cancer* 73:1049–1952.


Send reprint requests to: Dr. Stefania D'Atri, Istituto Dermopatico Dell'Immacolata (IDI-IRCCS), Via dei Monti di Creta 104, 00167 Rome, Italy. E-mail: s.datri@idi.it