A single cycle of treatment with temozolomide, alone or combined with O^6-benzylguanine, induces strong chemoresistance in melanoma cell clones *in vitro*: Role of O^6-methylguanine-DNA methyltransferase and the mismatch repair system

ESTER ALVINO[1], DANIELE CASTIGLIA[2], SIMONA CAPORALI[3], RITA PEPPONI[3,6], PATRIZIA CAPORASO[3], PEDRO MIGUEL LACAL[3], GIANCARLO MARRA[4], FRANZISKA FISCHER[4], GIOVANNA ZAMBRUNO[2], ENZO BONMASSAR[5], JOSEPH JIRICNY[4] and STEFANIA D'ATRI[3]

[1]Institute of Neurobiology and Molecular Medicine, Tor Vergata Research Area, National Research Council, Via Fosso del Cavaliere 100, I-00133 Rome; Laboratories of [2]Molecular and Cell Biology and [3]Molecular Oncology, Istituto Dermopatico dell'Immacolata-IRCCS, Via dei Monti di Creta 104, I-00167 Rome, Italy; [4]Institute of Molecular Cancer Research, University of Zürich, Winterhurerstrasse 190, CH-8057 Zürich, Switzerland; [5]Department of Neurosciences, University of Tor Vergata, Via Montpellier 1, I-00133 Rome, Italy

Received February 1, 2006; Accepted April 14, 2006

Abstract. Clinically achievable concentrations of temozolomide (TMZ) produce cytotoxic effects only in mismatch repair (MMR)-proficient cells endowed with low O^6-methylguanine-DNA methyltransferase (MGMT) activity. Aim of the present study was to investigate the molecular mechanisms underlying acquired resistance of melanoma cells to TMZ and the effect of O^6-benzylguanine (BG), a specific MGMT inhibitor, on the development of a TMZ-resistant phenotype. Three MMR-proficient melanoma cell clones with low or no MGMT activity were treated daily for 5 days with 50 μmol/l TMZ, alone or in combination with 5 μmol/l BG. Parental clones and sublines established after one or four cycles of treatment were analyzed for sensitivity to TMZ or TMZ+BG and for other parameters. The sublines established after one cycle of TMZ or TMZ+BG exhibited a marked increase in MGMT activity and resistance to TMZ alone. BG only partially reversed acquired resistance to the drug. In some cases, alterations in the MMR system accounted for MGMT-independent resistance to TMZ. Up-regulation of MGMT activity was associated with either demethylation of the *MGMT* promoter or hypermethylation of the body of the gene, and partially reversed by 5-aza-2'-deoxycytidine. The sublines established after four cycles of TMZ or TMZ+BG did not show a further increase in resistance to TMZ alone. However, two out of three sublines established after TMZ+BG treatment exhibited increased resistance to TMZ+BG. In conclusion, our data demonstrate that a single cycle of TMZ is sufficient to induce high levels of drug resistance in melanoma clones, principally, but not exclusively, via up-regulation of *MGMT* expression. Exposure to TMZ+BG favors the development of MGMT-independent mechanisms of TMZ resistance.

Correspondence to: Dr Stefania D'Atri, Laboratory of Molecular Oncology, Istituto Dermopatico dell'Immacolata-IRCCS, Via dei Monti di Creta 104, I-00167 Rome, Italy
E-mail: s.datri@idi.it

Present address: [6]Department of Drug Research and Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome, Italy

Key words: O^6-methylguanine-DNA methyltransferase, mismatch repair, temozolomide, chemoresistance, melanoma

Introduction

Resistance to chemotherapeutic agents is a major obstacle for the successful treatment of cancer. Most tumors are intrinsically resistant to chemotherapy or develop resistance after an initial response. Acquired chemoresistance can result from both drug-induced selection of pre-existing resistant cell clones and drug-induced genetic and epigenetic alterations of neoplastic cells. Increasing our understanding of the mechanisms underlying primary and acquired drug resistance may lead to the development of more successful therapeutic strategies.

Metastatic melanoma commonly exhibits primary or acquired resistance to chemotherapy. The methylating agent dacarbazine is considered to be the most active drug for the treatment of this cancer, with a response rate of 15-20% (reviewed in ref. 1). A number of combination regimens which include dacarbazine increase this response rate (1). However, no combination drug therapy has yet been shown to improve the overall survival rate in comparison to dacarbazine alone (1).

Temozolomide (TMZ) is a methylating agent that spontaneously decomposes into 5-(3-methyl-1-triazeno)imidazole-4-carboxamide, the active metabolite of dacarbazine (2). The drug has recently been approved for the treatment of recurrent high-grade gliomas and is in phase II/III clinical trials for the treatment of melanoma and other solid neoplasias. TMZ has comparable activity to dacarbazine in melanoma (1). However, unlike dacarbazine, TMZ penetrates the blood-brain barrier, which can be beneficial in preventing or treating melanoma metastases to the central nervous system (1).

The cytotoxicity of TMZ, like that of dacarbazine, is primarily due to the methylation of the O^6 position of guanine (O^6-G) in DNA. This is supported by the inverse correlation existing between the sensitivity of tumor cell lines to TMZ and the activity of the DNA-repair protein O^6-methylguanine-DNA methyltransferase (MGMT, E.C.2.1.1.63), which removes small alkyl groups from O^6-G in DNA and accepts them via binding to an internal cysteine residue in a stoichiometric and auto-inactivating reaction (reviewed in refs. 3,4). Consistently, depletion of MGMT activity by the competitive inhibitor O^6-benzylguanine (BG) (reviewed in ref. 5) increases tumor cell sensitivity to TMZ both *in vitro* and *in vivo* (reviewed in refs. 6,7).

The cytotoxic effects generated by DNA O^6-methylguanine (O^6-MeG) rely on the formation of O^6-MeG:T and O^6-MeG:C mispairs in the course of DNA duplication and the subsequent engagement of the mismatch repair (MMR) system (reviewed in ref. 8). According to the 'futile repair' model (9) the MMR system recognizes and attempts to process O^6-MeG:T and O^6-MeG:C mispairs. However, since the modified base is in the template strand, and MMR targets the newly synthesized strand, this repair event results in the degradation of the pyrimidine-containing strand and the subsequent reinsertion of C or T opposite the O^6-MeG. Reiterated 'futile' attempts at repair actually lead to the formation of gaps in the newly-synthesized DNA, which are converted into DNA double strand breaks in the course of the subsequent S-phase (8-11). DNA damage produced by the unsuccessful processing of O^6-MeG:T and O^6-MeG:C mismatches activates a signaling cascade resulting in cell cycle arrest at the G_2 phase of the second cell doubling event (12-15). This is followed by either apoptosis (12,15), mitotic catastrophe, or a senescence-like state (13,16). According to the 'signaling' model (17) after the recognition of O^6-MeG:T and O^6-MeG:C mispairs, the MMR system transmits the damage signal directly to the checkpoint machinery, without the need for DNA processing. Cells with a defective MMR are highly resistant to TMZ and other O^6-G-methylating agents regardless of their MGMT activity, and BG fails to increase drug sensitivity in these cells (6,18).

Previous studies by our group and other authors (19-21) have shown that primary resistance of melanoma cells to TMZ is mainly, although not exclusively, dependent on either high MGMT levels or MMR-deficiency. The aim of this study was to gain insight into the molecular changes induced by TMZ in MMR-proficient melanoma cells, endowed with low or no MGMT activity, leading to increased resistance to the drug. To this end, clonal melanoma cell populations were subjected to *in vitro* TMZ treatment designed to mimic patient exposure to the drug. Moreover, we have considered that BG is presently under clinical investigation with the intent to attenuate tumor cell resistance to TMZ. Therefore, melanoma cell clones were also treated with TMZ along with BG in order to investigate how exposure to the triazene compound in the presence of stable impairment of MGMT activity could affect the development of a TMZ-resistant phenotype.

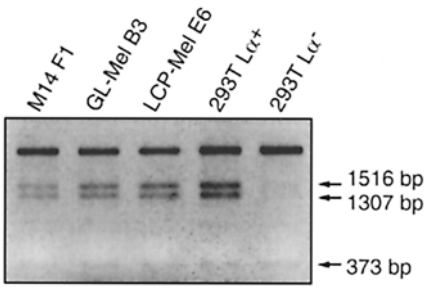

Figure 1. MMR proficiency of melanoma parental clones. Nuclear extracts of M14 F1, GL-Mel B3 and LCP-Mel E6 cells were assayed for the ability to repair G•T mismatches as described in Materials and methods. Nuclear extracts of the MMR-proficient 293T $L\alpha^+$ and MMR-deficient 293T $L\alpha^-$ cell lines were used as positive and negative control, respectively. The generation of the restriction fragments of 1516 and 1307 bp is indicative of G•T to A•T repair.

Materials and methods

Cell lines and clones. The human melanoma cell line M14 (22) was donated by Dr Gabriella Zupi (Regina Elena Cancer Institute, Rome, Italy), and the human melanoma cell lines LCP-Mel and GL-Mel (21) were kindly provided by Dr Fiorella Guadagni (Regina Elena Cancer Institute, Rome, Italy). LCP-Mel was derived from a primary melanoma, while M14 and GL-Mel were established from a cutaneous and lymph node metastasis, respectively. The three cell lines were previously shown to be MMR-proficient (21).

M14, LCP-Mel and GL-Mel were cloned by limiting dilution and a number of derived clones were tested for MGMT activity and sensitivity to TMZ. M14 F1, LCP-Mel E6 and GL-Mel B3 clones, endowed with very low (LCP-Mel E6 and GL-Mel B3) or no (M14 F1) MGMT activity were selected for this study. These three clones were confirmed to be MMR-proficient (Fig. 1).

The melanoma cell lines and clones were cultured at 37˚C in 5% CO_2 humidified atmosphere and maintained in RPMI-1640 (Hyclone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56˚C, 30 min) fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mmol/l L-glutamine and antibiotics (Invitrogen, Paisley, UK), hereafter referred to as complete medium (CM).

The MGMT-deficient human B lymphoblastoid cell line TK6 (23) was kindly provided by Dr W.G. Thilly (MIT, Cambridge, MA), and cultured in CM. The colon carcinoma cell lines LoVo and SW48 were obtained from the American Type Culture Collection (Rockville, MD), and cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine and antibiotics. SW48 cells are devoid of MGMT activity, while LoVo cells possess high MGMT levels (24,25).

The 293T Lα cell line was derived from the hMLH1-deficient human embryonic kidney 293T cells by stable transfection with a vector carrying the *hMLH1* cDNA under the control of the inducible Tet-Off expression system (26). The cell line was maintained in DMEM (containing 4500 mg/l glucose, 2 mmol/l L-glutamine and no pyruvate) (Invitrogen), supplemented with 10% Tet System approved fetal bovine serum (Clontech, Palo Alto, CA), 100 μg/ml zeocin (Invitrogen) and 300 μg/ml hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany). The cell line cultivated in the presence of doxycycline (0.05 μg/ml, Clontech) is MMR-deficient (293T Lα$^-$), while the cell line cultivated in the absence of doxycycline is MMR-proficient (293T Lα$^+$) (26).

Drugs, reagents and antibodies. TMZ was kindly provided by the Schering-Plough Research Institute (Kenilworth, NJ). BG, 5-aza-2'-deoxycytidine (5-AZA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma (St. Louis, MO).

TMZ to be utilized to generate TMZ-resistant melanoma sublines was dissolved in dimethyl sulfoxide (DMSO) (180 mmol/l), stored as a stock solution at -80˚C and diluted in CM just prior to use. TMZ to be utilized in the chemosensitivity assays was always freshly prepared in CM and discarded after use.

BG and 5-AZA were dissolved in ethanol (2.4 mg/ml) and 50% acetic acid (50 mmol/l), respectively, stored as stock solutions at -80˚C, and diluted in CM just prior to use. MTT was dissolved at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) and stored at 4˚C.

Mouse monoclonal antibodies (mAb) against hMSH2 (clone GB12) and hPMS2 (clone 9) were purchased from Oncogene Research Products (Boston, MA); mouse mAb against hMLH1 (clone G168-15) was obtained from BD PharMingen (Heidelberg, Germany); mouse mAb against hMSH6 (clone 44) was purchased from BD Transduction Laboratories (Heidelberg, Germany); polyclonal anti-hMSH3 rabbit antiserum was generated at Eurogentec (Herstal, Belgium) by immunization with a His 6-tagged N-terminal polypeptide of hMSH3 (amino acids 1-200) according to standard protocols; goat polyclonal antibody against MGMT (C20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). mAb against actin (clone AC-40) and horseradish peroxidase-linked rabbit anti-goat IgG came from Sigma; horseradish peroxidase-linked sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from Amersham Biosciences (Little Chalfont, UK).

All reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences.

Generation of TMZ-resistant melanoma sublines. The recommended dosage of TMZ is 200 mg/m^2/day for five consecutive days, to be repeated every four weeks. The peak plasma concentration achieved for each drug administration is approximately 50 μmol/l (27).

To reproduce *in vitro* the clinical application of TMZ, M14 F1, LCP-Mel E6, and GL-Mel B3 cell clones were suspended in CM at a concentration of 1x10^4 cells/ml, dispensed in 4 ml aliquots into flat-bottom 6-well plates (Falcon, Becton and Dickinson Labware, Franklin Lakes, NJ) and allowed to adhere over 6 h at 37˚C. An appropriate amount of TMZ was then added to the wells to obtain a final drug concentration of 50 μmol/l. Control groups were treated with DMSO alone. The plates were incubated at 37˚C in a 5% CO_2 humidified atmosphere. Cell treatment was repeated every 24 h for a total of five exposures to TMZ or DMSO. TMZ treatment was also performed in the presence of 5 μmol/l BG. In this case, the MGMT inhibitor was added to the wells 2 h before TMZ and left in the cultures for the entire cycle of treatment. Control groups were treated with BG+DMSO alone.

All treated cells were maintained in culture until exponential growth of TMZ-treated cultures resumed. Each culture was then divided into two aliquots: one aliquot was cultured in drug-free CM as an independent melanoma subline, while the other was subjected to a new cycle of treatment with TMZ, TMZ+BG, DMSO or DMSO+BG. This procedure was repeated for a total of four cycles of treatment.

Western blot analysis. Cells were suspended in lysis-buffer [50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EGTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l AEBSF, and 1X protease inhibitor cocktail Complete EDTA-free (Roche Diagnostics)] for 10 min on ice. Cell lysates were then clarified by centrifugation, diluted in 5X Laemmli sample buffer and boiled for 5 min. Sixty μg of protein per sample were run on 7% (MMR proteins) or 15% (MGMT protein) SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences), and blocked in 50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 0.1% Tween-20, and 5% (MMR proteins) or 10% (MGMT protein) non-fat dry milk for 1 h at 37˚C. The membranes were then incubated in the same solution overnight at 4˚C with the following primary antibodies: anti-hMSH2 (1 μg/ml), anti-hMSH3 (1:6000), anti-hMSH6 (0.5 μg/ml), anti-hMLH1 (2 μg/ml), anti-PMS2 (0.5 μg/ml), anti-MGMT (1 μg/ml) and anti-actin (1:1000). The latter mAb was used as an internal standard for loading. Immunodetection was carried out by using appropriate horseradish peroxidase-linked secondary antibodies and ECL detection reagents.

Preparation of nuclear extracts for in vitro MMR assay. Exponentially multiplying melanoma cells were washed in PBS and used for preparation of nuclear extracts as described by Holmes *et al* (28) with minor modifications. The isolated nuclei were resuspended in cold extraction buffer [25 mmol/l HEPES-KOH, pH 7.5, 10% sucrose, 1 mmol/l phenylmethylsulfonyl fluoride, 0.5 mmol/l dithiothreitol, 1 μg/ml leupeptin, and 1 of tablet protease inhibitor (Complete Mini, Roche Diagnostics)] in the smallest volume possible. NaCl (5 mol/l, 0.031 Vol) was then added and the mixture was incubated with rolling for 1 h at 4˚C (final concentration NaCl: 0.155 mol/l). The nuclear debris was pelleted at 14,500 x g for 20 min at 2˚C. The supernatant was dialysed for 2 h at 4˚C against dialysis buffer (25 mmol/l HEPES-KOH, pH 7.5, 50 mmol/l KCl, 0.1 mmol/l EDTA, pH 8.0, 10% sucrose, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l dithiothreitol,

and 1 μg/ml leupeptin). Following dialysis, the extract was clarified by centrifugation at 20,000 x g for 15 min at 2˚C. The nuclear extract was then stored in small aliquots at -80˚C. The conductivity of the extracts was measured and the salt concentration was calculated based on a standard KCl concentration curve.

Construction of the MMR substrate. A circular DNA heteroduplex (3.195 kb) suitable for the *in vitro* MMR reaction was constructed by primer extension using single-stranded DNA and the phosphorylated oligonucleotide 5'-CCAGACGTCT GTCGACGTTGGGAAGCTTGAG-3' (Microsynth GmbH, Balgach, Switzerland). The pGEM13Zf(+) ssDNA, with a modified linker sequence, was isolated from the XL1Blue *E. coli* strain transformed with pGEMA•T and superinfected with M13KO7 helper phage. In brief, 60 μg (57 pmoles) of this ssDNA were annealed with 2.1-fold molar excess of phosphorylated oligonucleotide (120 pmoles) in 240 μl of T4 DNA Polymerase reaction buffer [50 mmol/l Tris-HCl, 15 mmol/l $(NH_4)SO_4$, 7 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 10 mmol/l 2-mercaptoethanol, 0.02 mg/ml bovine serum albumin (BSA), pH 8.8 (25˚C)]. Extension was done at 37˚C for 1 h in a final volume of 600 μl by adding 100 μg/ml BSA, 1 mmol/l dNTPs, 1 mmol/l ATP, 54U of T4 DNA Polymerase and 2400 U T4 DNA ligase. The enzymes were inactivated by incubation at 70˚C for 20 min. After purification on a CsCl gradient the circular plasmid was nicked with the enzyme N.*Bst*NBI and purified using the Qiagen MinElute™ Reaction Cleanup Kit (Qiagen GmbH, Hilden, Germany). The resulting heteroduplex DNA contained a unique G•T mismatch located 350 bp downstream of the single strand incision.

MMR assay. The assays were performed as previously described (28) with minor modifications. The reaction mixtures (20 μl) contained 0.02 mol/l Tris-HCl, pH 7.6, 5 mmol/l $MgCl_2$, 1 mmol/l glutathione, 50 μg/ml BSA, 0.1 mmol/l of each dNTP, 1.5 mmol/l ATP, 110 mmol/l KCl, 0.1 μg of heteroduplex DNA and 75 μg of nuclear cell extract. Reactions were incubated at 37˚C for 45 min and terminated by the addition of 30 μl of stop solution (25 mmol/l EDTA, 0.67% SDS, 50 μg/ml proteinase K) for 30 min at 37˚C. After purification using the Qiagen MinElute Reaction Cleanup Kit the DNA samples were treated with the *Acl*I restriction enzyme and run on a 1% agarose gel in TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA, pH 8.0). In the absence of repair the pGEM phagemid is cleaved by *Acl*I into two fragments of 373 and 2823 bp. The presence of three digested fragments of 1516, 1307 and 373 bp is indicative of G•T to A•T repair.

Northern blot analysis. Total RNA was isolated from melanoma cell lines using a standard method. For each sample, 20 μg of total RNA were fractionated by electrophoresis on a 1.2% agarose/formaldehyde gel and transferred to Hybond-N^+ nylon membrane (Amersham Biosciences) as devised by the supplier. The quantity and integrity of RNA in each sample was confirmed by RNA visualization following staining with ethidium bromide (0.5 μg/ml). To detect the *MGMT* mRNA, membranes were hybridized with a [^{32}P]-labeled *MGMT* cDNA probe generated as previously described (29). Filters were exposed to autoradiographic film for five days.

Methylation-specific PCR (MSP). Genomic DNA was isolated using the DNeasy tissue kit (Qiagen) and subsequently treated (1 μg) with sodium bisulfite as described by Herman *et al* (30). DNA methylation status in a CpG-rich region of the *MGMT* promoter (31) was determined by subsequent PCR (32). The primer pair for the unmethylated DNA covered nt +110 to nt +202 with respect to the transcription start site, while the primer pair for the methylated DNA covered nt +116 to nt +196. PCR amplification of DNA was performed with an initial denaturation step of 10 min at 94˚C linked to 35 cycles for 30 sec at 94˚C, 30 sec at 59˚C and 30 sec at 72˚C, followed by a final extension step of 7 min at 72˚C. The PCR products (93 bp for unmethylated DNA and 81 bp for methylated DNA) were separated by electrophoresis on 2% agarose gel containing ethidium bromide and visualized under UV illumination. DNA extracted from the SW48 cell line was used as a positive control for methylated templates, whereas DNA extracted from the LoVo cell line was used as a negative control (25).

Sodium bisulfite DNA sequencing and combined bisulfite restriction analysis (COBRA). Genomic DNA was isolated and treated with sodium bisulfite as described above. Bisulfite-modified DNA was then amplified using the following primers, which encompass the third exon (149 bp) of the *MGMT* gene and part of its adjacent introns: 5'-TGTTGTATAGTTAGTT GAGATGTG-3' (F1, positioned 140 bp upstream from the 5' boundary of exon 3) and 5'-CCAAAAATTATACAACCCT ATACC-3' (R, positioned 215 bp downstream from the 3' boundary of exon 3). PCR amplification, which generates a 504 bp product, was performed in a reaction volume of 50 μl containing standard concentrations of the Ampli*Taq* Gold PCR reagents and 2.0 U of *Taq* Gold polymerase (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ). PCR conditions were as follows: an initial denaturation step of 10 min at 94˚C; 40 cycles for 1 min at 94˚C, 1 min at 57˚C, 1 min at 72˚C; a final extension step of 10 min at 72˚C. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) and directly sequenced on an ABI PRISM 377-96 DNA sequencer (Applied Biosystems, Foster City, CA) using the sense primer. PCR products were also cloned into the pCR2.1 TA vector (Invitrogen, San Diego, CA) and 10 individual clones per sample were sequenced as above.

For COBRA (33) bisulfite-modified DNA was initially amplified with primers F1 and R as described above. To improve the yields of the PCR products, a second round of PCR was performed with the primer R and the following nested forward primer (F2): 5'-GTGTTTATGAAGTAGTT ATAGGTG-3', which generated a 479-bp product. PCR conditions were as described for bisulfite sequencing for 31 cycles. PCR products were digested with *Taq*I (Roche Diagnostics) for 4 h at 65˚C, subjected to electrophoresis on 2% agarose gels containing ethidium bromide and visualized under UV illumination. The presence and number of digested bands were considered as an index of the methylation levels in the original DNA sample.

MGMT assay. Melanoma cells were removed from continuous culture, washed twice with PBS and stored as pellets at -80˚C until used. MGMT activity in cell extracts was determined by

measuring the transfer of [^{3}H]-methyl groups from a DNA substrate to the MGMT protein as previously described (21). MGMT activity was expressed in terms of fmoles of [^{3}H]-methyl groups transferred per mg of protein in cell extract. Statistical analysis on MGMT activity was performed according to Student's t-test.

Evaluation of cell sensitivity to TMZ by the MTT assay. Melanoma cells were suspended in CM at a concentration of $2x10^4$ cells/ml, dispensed in 50 μl aliquots into flat-bottom 96-well plates (Falcon) and allowed to adhere overnight at 37˚C. Graded amounts of TMZ were then added to the wells in 50 μl of CM and the plates were incubated at 37˚C in a 5% CO_2 humidified atmosphere for five days. Four replicate wells were used for controls and each drug concentration. The cytotoxic effects of TMZ were also evaluated in combination with the MGMT inhibitor BG. To this end, 5 μmol/l BG was added to the plates 2 h before TMZ and left in culture for the entire period of cell exposure to the drug. Control groups were either untreated or treated with BG alone.

The MTT assay was performed as previously described (21). Briefly, after five days of culture, 0.1 mg of MTT (in 20 μl of PBS) was added to each well and cells were incubated at 37˚C for 4 h. Cells were then lysed with a buffer (0.1 ml/well) containing 20% SDS and 50% N,N-dimethylformamide, pH 4.7. After overnight incubation, the absorbance was read at 595 nm using a 3550-UV microplate reader (Bio-Rad).

Cell sensitivity to drug treatment was expressed in terms of IC_{50} (drug concentration producing 50% inhibition of cell growth, calculated on the regression line in which absorbance values at 595 nm were plotted against the logarithm of drug concentration). Statistical analysis on IC_{50} values was performed according to Student's t-test.

Treatment with 5-AZA. Exponentially multiplying cells were seeded in 150-mm dishes (Falcon) ($2x10^6$ cells/dish, in 40 ml of CM) and allow to adhere 6 h at 37˚C in a CO_2 incubator. 5-AZA was then added to the cultures at the final concentration of 1 μmol/l. After 24-h incubation at 37˚C, culture supernatants were removed and fresh medium containing 1 μmol/l 5-AZA was added to the cultures. This procedure was repeated after an additional 24 h at 37˚C. Control groups were similarly treated with appropriate volumes of vehicle alone. Cells were recovered 48 h after the last treatment, washed in PBS and analyzed for MGMT activity and methylation status of the promoter and/or body of the *MGMT* gene.

Results

A single cycle of treatment with TMZ or TMZ+BG is sufficient to induce a marked increase of resistance to TMZ in melanoma cell clones. To gain insight into the molecular changes induced in melanoma cells by TMZ exposure leading to increased resistance to the drug, we used three melanoma cell clones, each derived from a different melanoma cell line. The clones were selected in order to provide *in vitro* models of MMR-proficient melanoma cells endowed with low or no MGMT activity showing different sensitivities to TMZ.

The cell clones were treated daily for five days with 50 μmol/l TMZ (hereafter referred to as 'cycle of treatment'), alone or in the presence of 5 μmol/l BG. Control groups were treated with DMSO or DMSO+BG. When TMZ-treated cultures resumed from drug-induced inhibition of cell growth, a new cycle of treatment was performed, for a total of four cycles. Melanoma sublines obtained after one cycle of treatment were identified with the name of the parental clone, followed by the type of treatment and the code 01 (e.g. M14 F1 TMZ/BG/01). Melanoma sublines obtained after four cycles of treatment were identified as above using the code 04. The parental clones and the corresponding sublines generated after one or four cycles of DMSO, DMSO+BG, TMZ or TMZ+BG were comparatively analyzed for sensitivity to TMZ alone or combined with BG.

The results illustrated in Table I show that the M14 F1 clone, devoid of MGMT activity, was the most susceptible to TMZ and, as expected, its sensitivity to the drug was not affected by the MGMT inhibitor. In the absence of BG, LCP-Mel E6 and GL-Mel B3 clones, endowed with low and comparable MGMT activity, displayed TMZ IC_{50} values significantly higher than those shown by M14 F1 cells. GL-Mel B3 was also significantly more resistant to TMZ than LCP-Mel E6. In both clones, BG significantly increased the inhibitory effect of TMZ on cell growth. However, while TMZ sensitivity of LCP-Mel E6 became comparable to that of M14 F1 clone, TMZ sensitivity of GL-Mel B3 clone remained significantly lower than that displayed by the other two clones. This finding indicates that GL-Mel B3 cells possess MMR- and MGMT-independent mechanisms of resistance to TMZ.

Sensitivity to TMZ or TMZ+BG of DMSO and DMSO/BG sublines, established after one or four cycles of treatment, was comparable to that of the corresponding parental clones (data not shown). Sensitivity of TMZ and TMZ/BG sublines is reported in Table I. In the absence of BG, all TMZ and TMZ/BG sublines displayed TMZ IC_{50} values significantly higher that those displayed by the corresponding parental clones. Notably, a marked increase of resistance to TMZ was also observed in the subline established from the GL-Mel B3 clone, that is already endowed with an appreciable level of resistance to the drug. TMZ and TMZ/BG sublines derived from the same parental clone showed comparable levels of drug resistance. Moreover, TMZ/04 and TMZ/BG/04 sublines exhibited TMZ IC_{50} values comparable to those displayed by the matched TMZ/01 and TMZ/BG/01 sublines. In the presence of BG, TMZ sensitivity of all TMZ and TMZ/BG sublines was significantly enhanced. However, BG treatment only partially reversed the acquired TMZ resistance. As observed in the absence of BG, the TMZ/01 and TMZ/BG/01 sublines derived from the same parental clone showed comparable resistance to TMZ. In contrast, two out of the three TMZ/BG/04 sublines (i.e. those derived from M14 F1 and LCP-Mel E6 clones) exibited TMZ IC_{50} values significantly higher than those displayed by the matched TMZ/04 sublines, or the matched TMZ/01 and TMZ/BG/01 sublines.

MGMT expression is upregulated by TMZ treatment. The finding that BG was able to partially reverse the acquired resistance to TMZ in all TMZ and TMZ/BG sublines strongly indicated that TMZ treatment could have induced an increase of MGMT activity in these sublines. We therefore evaluated

Table I. Chemosensitivity of melanoma parental clones and matched TMZ and TMZ/BG sublines.

		IC_{50} (μmol/l)[b]				
		TMZ		TMZ+BG		
Cell line	Cycles[a]	AM ± SE	P1[c]	AM ± SE	P1	P2[d]
LCP-Mel E6	-	81±5	-	35±3	-	0.01
GL-Mel B3	-	232±9	-	174±13	-	0.05
M14 F1	-	29±3	-	28±2	-	NS
LCP-Mel E6/TMZ/01	1	454±18	-	124±14	-	<0.01
LCP-Mel E6/TMZ/BG/01	1	467±29	NS	153±20	NS	<0.01
GL-Mel B3/TMZ/01	1	549±32	-	389±41	-	<0.01
GL-Mel B3/TMZ/BG/01	1	579±23	NS	450±32	NS	<0.01
M14 F1/TMZ/01	1	244±24	-	102±10	-	<0.01
M14 F1/TMZ/BG/01	1	248±22	NS	101±9	NS	<0.01
LCP-Mel E6/TMZ/04	4	483±57	-	147±18	-	<0.01
LCP-Mel E6/TMZ/BG/04	4	528±40	NS	289±7[e]	<0.01	<0.01
GL-Mel B3/TMZ/04	4	597±57	-	447±42	-	<0.05
GL-Mel B3/TMZ/BG/04	4	588±49	NS	437±33	NS	<0.05
M14 F1/TMZ/04	4	288±35	-	107±18	-	<0.01
M14 F1/TMZ/BG/04	4	295±20	NS	202±8[e]	<0.05	<0.01

[a]Number of cycles of TMZ or TMZ+BG treatment used to generate the indicated sublines. [b]Cells were incubated with graded concentrations of TMZ alone or combined with 5 μmol/l BG, for 5 days and then analyzed for cell growth by the MTT assay. Exposure to BG was performed by incubating cells with BG for 2 h before treatment with TMZ and maintaining the inhibitor in culture until the end of the assay. Cell sensitivity to drug treatment is expressed in terms of IC_{50}, i.e, drug concentration required to inhibit cell growth by 50%. Each value represents the arithmetic mean ± standard error of at least three independent experiments. [c]P1, probability calculated according to Student's t-test comparing the IC_{50} values of TMZ/BG sublines with those of the matched TMZ sublines. NS, not significant. [d]P2, probability calculated according to Student's t-test comparing for each cell line the IC_{50} values obtained in the presence of BG with those obtained without the inhibitor. [e]$P<0.01$, according to Student's t-test comparing the IC_{50} values of the sublines generated after four cycles of treatment with those of the matched sublines generated after one cycle of treatment.

MGMT activity in TMZ/01 and TMZ/BG/01 sublines and, as a control, in the matched DMSO/01 and DMSO/BG/01 sublines. As illustrated in Fig. 2a, MGMT activity of DMSO/01 and DMSO/BG/01 sublines was comparable to that of the corresponding parental clone. MGMT activity of TMZ/01 and TMZ/BG/01 sublines was, instead, significantly higher than that of the matched parental clone. No decrease in MGMT activity was detected in the TMZ/01 and TMZ/BG/01 sublines cultivated for up to 6 months in the absence of selective pressure (data not shown).

To investigate whether the increase of MGMT activity observed in the TMZ/01 and TMZ/BG/01 sublines was due to increased protein levels, total cell extracts were subjected to Western blot analysis using an MGMT specific polyclonal antibody. MGMT expression was very low in LCP-Mel E6 and GL-Mel B3 parental clones as well as in their matched DMSO/01 and DMSO/BG/01 sublines (Fig. 2b). No MGMT expression was detected in M14 F1 parental clone and its DMSO/01 and DMSO/BG/01 sublines, while MGMT protein levels were markedly increased in all TMZ/01 and TMZ/BG/01 sublines (Fig. 2b).

We then evaluated whether the increase of MGMT protein detected in the TMZ/01 and TMZ/BG/01 sublines was due to increased levels of the MGMT gene transcript. To this end, total RNA purified from each parental clone and matched sublines was subjected to Northern blot analysis with a radioactively labelled *MGMT* cDNA probe. Parental LCP-Mel E6 and GL-Mel B3 clones and their matched DMSO/01 and DMSO/BG/01 sublines displayed very low levels of the *MGMT* transcript, while no transcript was detected in the M14 F1 clone and its DMSO/01 and DMSO/BG/01 sublines (Fig. 2c). Conversely, all TMZ/01 and TMZ/BG/01 sublines exhibited an increase in the levels of the *MGMT* transcript with respect to their corresponding parental clone (Fig. 2c).

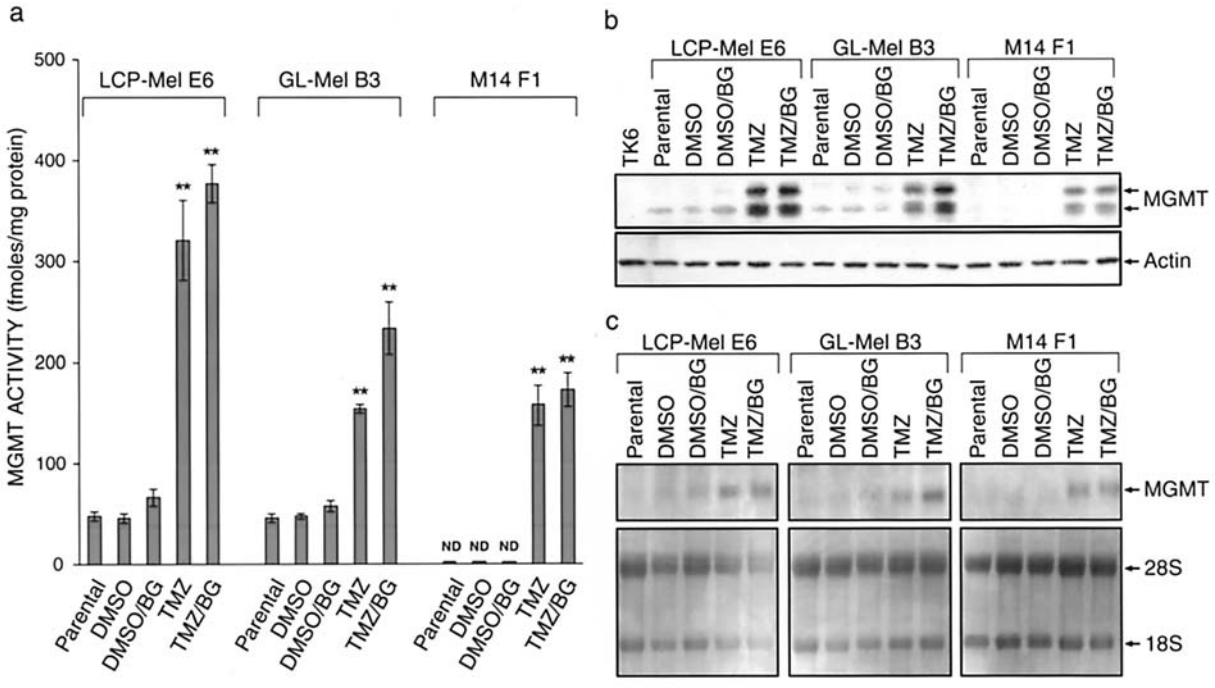

Figure 2. MGMT activity, protein and mRNA expression in parental melanoma clones and matched sublines established after one cycle of treatment with TMZ, TMZ+BG, DMSO or DMSO+BG. (a) MGMT activity is expressed in terms of fmoles of methyl groups removed per mg protein in cell extracts. Each value represents the arithmetic means of at least three independent experiments. Bars, standard error of the mean. ND, not detectable. $^{**}P<0.01$, according to Student's t-test comparing MGMT activity of each subline with that of the corresponding parental clone. (b) Sixty μg of whole cell extracts were subjected to electrophoresis on a 15% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with a polyclonal antibody against MGMT. Incubation with anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. The MGMT-deficient TK6 cell line was used as a negative control. The presence of double protein bands probably reflects the existence of differentially phosphorylated forms of MGMT, as previously reported. (c) Total cellular RNA (20 μg per lane) was size fractionated on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane and hybridized with a [^{32}P]-labelled *MGMT* cDNA probe (upper panels). Ethidium bromide staining of 28S and 18S ribosomal RNA was performed as a loading control (lower panels).

TMZ treatment induces changes in the methylation pattern of the MGMT gene. Previous studies have shown that the expression of the *MGMT* gene can be affected by CpG methylation. Methylation can occur in both the promoter and the body of the *MGMT* gene, resulting in opposite effects. Methylation of the promoter is usually associated with the silencing of the gene, while methylation outside the promoter is associated with increased transcription (3,4).

To investigate the methylation status of the *MGMT* gene in the three parental clones and in the corresponding TMZ/01 and TMZ/BG/01 sublines, genomic DNA was isolated from the cells and subjected to sodium bisulfite modification. MSP was then used to analyze an *MGMT* promoter-associated CpG-rich region previously shown to be hypermethylated in MGMT-deficient cell lines and unmethylated in MGMT-expressing cell lines (25,31). The results illustrated in Fig. 3a show that in the LCP-Mel E6 parental clone and its TMZ/01 and TMZ/BG/01 sublines the *MGMT* promoter was unmethylated. Similar results were obtained in the GL-Mel B3 parental clone and matched TMZ/01 and TMZ/BG/01 sublines (data not shown). Only methylated alleles of the *MGMT* promoter were detected in the M14 F1 clone, while in its TMZ/01 and TMZ/BG/01 sublines both methylated and unmethylated alleles were observed (Fig. 3a). No changes in the methylation status of the *MGMT* promoter occurred in the DMSO/01 or DMSO/BG/01 sublines derived from the three different clones (data not shown).

The methylation status of the body of the *MGMT* gene was determined by sodium bisulfite DNA sequencing. The area investigated spans exon 3 and part of its adjacent introns and contains 21 CpG sites (Fig. 3b, top). This region (504 bp) was amplified from bisulfite-treated DNA using intronic primers. The methylation status of each CpG site was determined by both direct sequencing of the PCR products and sequencing of 10 independent single-copy DNAs generated by cloning the PCR products into a plasmid vector.

In LCP-Mel E6 parental clone the majority of the CpG sites under investigation were unmethylated in every *MGMT* allele examined (Fig. 3b, right panel). In contrast, in the TMZ/01 and TMZ/BG/01 sublines derived from this clone almost all the CpG sites were methylated in every allele (Fig. 3b, right panel). Similar results were obtained in GL-Mel B3 parental clone and matched TMZ/01 and TMZ/BG/01 sublines (data not shown).

In M14 F1 parental clone only 2 out of 21 CpG positions were unmethylated in all the *MGMT* alleles examined, while the remaining CpG sites were methylated in almost all alleles (Fig. 3b, right panel). M14 F1-derived TMZ/01 and TMZ/BG/01 sublines exhibited changes in the pattern of CpG methylated sites, with an overall slight decrease of methylation density (Fig. 3b, right panel).

A representative chromatogram of the direct sequence encompassing CpG sites 4-7, as determined in LCP-Mel parental clone and the corresponding TMZ/01 subline, is illustrated in the left panel of Fig. 3b.

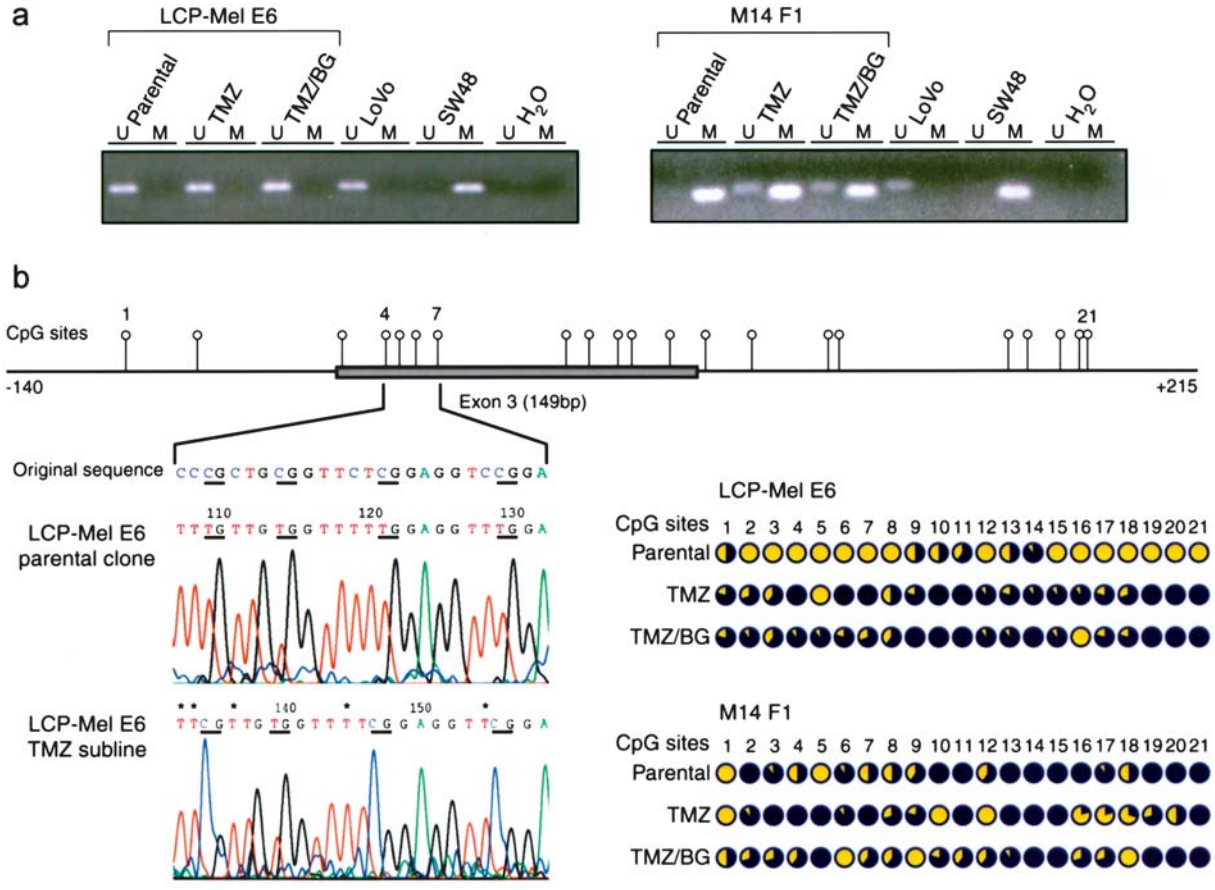

Figure 3. Analysis of the methylation status of the *MGMT* gene in parental melanoma clones and matched sublines established after one cycle of treatment with TMZ or TMZ+BG. (a) MSP analysis of *MGMT* promoter. Genomic DNA was extracted from parental melanoma clones and their corresponding TMZ/01 and TMZ/BG/01 sublines and treated with sodium bisulfite. Paired samples of bisulfite-treated DNA were then subjected to PCR amplification of a CpG-rich region of the *MGMT* promoter using primers specific for either unmethylated (U) or methylated (M) DNA alleles. The colon carcinoma cell lines LoVo and SW48 served as controls for unmethylated DNA and methylated DNA, respectively. (b) Sodium bisulfite DNA sequence analysis of the body of the *MGMT* gene. Top, schematic representation of the *MGMT* gene region analyzed (504 bp) illustrating the positions of the 21 CpG sites within this region. Left panel, sequence chromatograms of the exon 3 region spanning CpG sites 4-7, following sodium bisulfite conversion of the DNA extracted from LCP-Mel E6 parental clone and its TMZ/01 subline. In the parental clone all cytosine residues were converted to thymine. In the TMZ/01 subline all cytosine residues at CpG sites, except site 5, remained as cytosine. For comparison, the original sequence of the region is also shown. *Cytosine in non-CpG sites converted to thymine. Right panel, the methylation status of each CpG site in parental melanoma clones and matched TMZ/01 and TMZ/BG/01 sublines was determined through the sequencing of 10 independent single-copy DNAs generated by cloning the 504 bp PCR product into the pCR2.1 TA vector. Yellow circles, CpG sites unmethylated in all the clones sequenced; blue circles, CpG sites methylated in all the clones sequenced; partially-blue circles, CpG sites methylated in a proportion of the clones sequenced: the degree to which the circle is filled in blue is directly related to the number of clones with the methylated allele.

Effects of 5-AZA treatment on MGMT activity and the methylation status of the MGMT gene. The analysis of the methylation status of the *MGMT* gene performed in LCP-Mel E6 and GL-Mel B3 cell clones and the derived TMZ/01 and TMZ/BG/01 sublines indicated that in the drug-treated sublines the increase of MGMT activity was accompanied by hypermethylation of the body of the *MGMT* gene. To establish whether this hypermethylation was causally related to the increase in MGMT activity, the LCP-Mel E6 TMZ/01 subline was treated with 1 μmol/l 5-AZA, a drug known to lower the level of CpG methylation, and then assayed for the methylation status of the body of the *MGMT* gene and for the enzyme activity.

In the original sequence of the *MGMT* gene body region analyzed in this study there is one *Taq*I restriction site (TCGA), corresponding to CpG site 9 (Fig. 4a). Upon treatment of DNA with sodium bisulfite the restriction site is retained only if the CpG position is methylated. Moreover, three additional *Taq*I restriction sites, corresponding to CpG positions 2, 8 and 18, are newly formed after sodium bisulfite treatment if the CpG sites are methylated (Fig. 4a). Therefore, possible changes in the methylation status of the body of the *MGMT* gene induced by 5-AZA treatment were evaluated by COBRA.

The results illustrated in Fig. 4b show that in the LCP-Mel E6 TMZ/01 subline 5-AZA induced a demethylation of the body of the *MGMT* gene. Indeed, the number of restricted bands generated by *Taq*I digestion of the 479-bp fragment amplified from bisulfite-treated DNA was reduced in the cells exposed to 5-AZA with respect to the cells treated with acetic acid alone (the solvent use for 5-AZA). Demethylation of the body of the *MGMT* gene was accompanied by a reduction of MGMT activity [(358±11 and 196±12 fmoles/mg protein in control and 5-AZA-treated cells, respectively ($P<0.01$)].

In the TMZ/01 and TMZ/BG/01 sublines derived from the M14 F1 clone the increase in MGMT activity was accompanied by a partial demethylation of the *MGMT* promoter. We

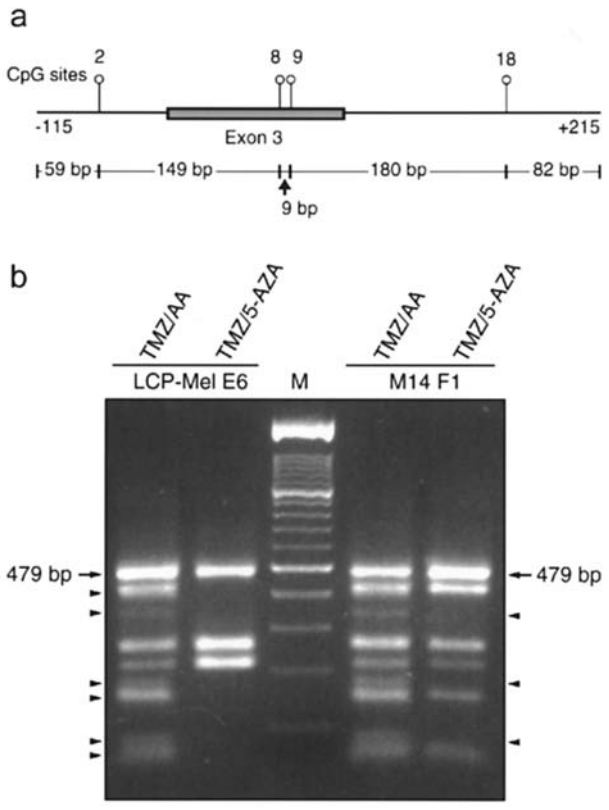

Figure 4. Changes in the methylation status of the body of the *MGMT* gene induced by 5-AZA in TMZ/01 sublines derived from LCP-Mel E6 and M14 F1 parental clones. (a) Schematic representation of the region of the body of the *MGMT* gene subjected to COBRA. The CpG sites generating *Taq*I restriction sites (TCGA) if methylated, and the size of the *Taq*I restricted fragments are shown. (b) TMZ/01 sublines derived from LCP-Mel E6 and M14 F1 clones were treated with 1 μmol/l 5-AZA (TMZ/5-AZA) or appropriate amounts of 50% acetic acid (TMZ/AA) as described in Materials and methods. Genomic DNA was then extracted from the cells and the methylation status of the *MGMT* region illustrated in (a) was determined by COBRA. Bisulfite-modified DNA was subjected to a first round of PCR using primers F1 and R, and to a second round of PCR using primers R and F2. The 479 bp PCR product was digested with *Taq*I, subjected to electrophoresis on 2% agarose gel containing ethidium bromide and visualized under UV illumination. Arrowheads, restricted bands present in acetic acid-treated but not in 5-AZA-treated cells. M, 100-bp DNA ladder (Marker XIV, Roche Molecular Biochemicals).

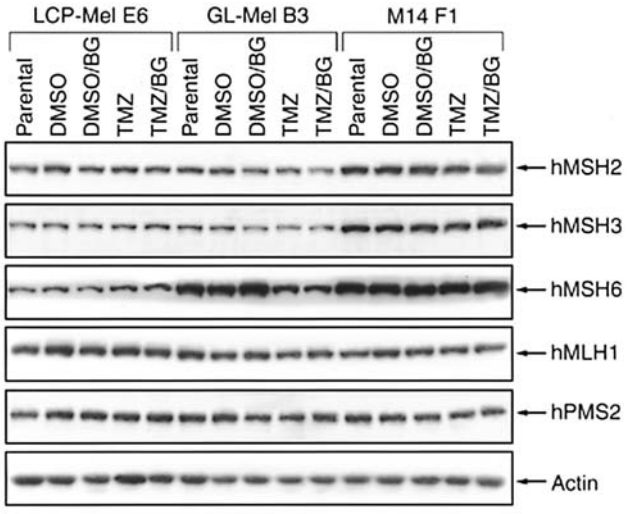

Figure 5. MMR protein expression in parental melanoma clones and matched sublines established after one cycle of treatment with TMZ or TMZ+BG. Sixty μg of whole cell extract were subjected to electrophoresis on a 7% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with antibodies against hMSH2, hMSH3, hMSH6, hMLH1 or hPSM2. Incubation with anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL.

therefore sought to treat the M14 F1 TMZ/01 subline with 5-AZA to investigate how a further demethylation of the *MGMT* gene would affect the enzyme activity. The subline was treated with acetic acid or 1 μmol/l 5-AZA and then analyzed for the methylation status of both the promoter and body of the *MGMT* gene, as well as for MGMT activity.

In M14 F1 TMZ/01 cells 5-AZA treatment induced a demethylation of the body of the *MGMT* gene (Fig. 4b) and a decrease of MGMT activity [(165±8 and 66±6 fmoles/mg protein in control and 5-AZA-treated cells, respectively ($P<0.01$)]. MSP analysis of the gene promoter revealed the presence of both methylated and unmethylated alleles in control and 5-AZA-treated cells (data not shown).

Expression of MMR proteins in TMZ/01 and TMZ/BG/01 sublines. TMZ/01 and TMZ/BG/01 sublines displayed TMZ IC_{50} values significantly higher than those exhibited by the corresponding parental clones even when drug sensitivity was evaluated in the presence of BG (Table I). This finding strongly indicated that up-regulation of MGMT activity was not the only TMZ-induced molecular change underlying the acquired TMZ-resistance of these sublines.

We have previously shown that when MGMT activity is inhibited with BG, TMZ sensitivity of melanoma cells is largely, although not exclusively, dictated by their MMR efficiency (21). Moreover, an inverse correlation has been demonstrated between cellular amounts of the hMSH2 protein and the level of resistance to the methylating drug (34). We therefore investigated whether the TMZ/01 and TMZ/BG/01 sublines displayed reduced expression of MMR proteins. To this end, total cell extracts were subjected to Western blot analysis using antibodies against the hMSH2, hMSH3, hMSH6, hMLH1 and hPMS2 proteins.

The TMZ/01 and TMZ/BG/01 sublines derived from GL-Mel B3 clone displayed reduced expression of hMSH6 protein with respect to the parental clone. No substantial changes in the levels of the MMR proteins were observed in the other sublines (Fig. 5).

Exposure to TMZ may lead to inactivation of MMR. To investigate the reproducibility of TMZ-induced up-regulation of MGMT activity, each parental melanoma clone was subjected to six additional independent exposures to one cycle of TMZ or TMZ+BG treatment. The newly established TMZ/01 and TMZ/BG/01 sublines were then assayed for MGMT activity. All drug-treated sublines showed increased MGMT activity with respect to their matched parental clone with the exception of one TMZ/BG/01 subline derived from the M14 F1 clone (data not shown). This subline (hereafter referred to as TMZ/BG/MGMT$^-$) was, like the parental clone, devoid of MGMT activity. In agreement with this finding, we were unable to detect MGMT protein and transcript in TMZ/BG/MGMT$^-$ cells (Fig. 6a and b). Moreover, in these

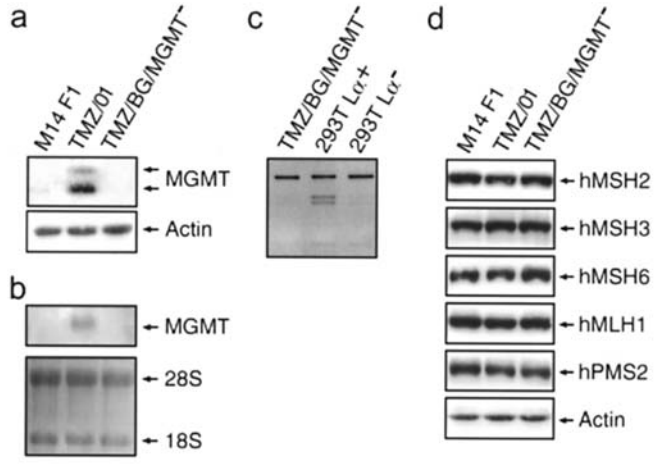

Figure 6. Characterization of the TMZ/BG/MGMT- subline derived from M14 F1 parental clone. (a) Sixty μg of whole cell extracts obtained from M14 F1 parental clone and the derived TMZ/BG/MGMT- subline were subjected to electrophoresis on a 15% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with a polyclonal antibody against MGMT. Incubation with anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. A TMZ/01 subline derived from M14 F1 clone was used as a positive control. (b) Twenty μg of total RNA obtained from M14 F1 parental clone and the derived TMZ/BG/MGMT- subline were size fractionated on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane and hybridized with a [^{32}P]-labelled *MGMT* cDNA probe (upper panel). Ethidium bromide staining of 28S and 18S ribosomal RNA was performed as a loading control (lower panel). A TMZ/01 subline derived from M14 F1 clone was used as a positive control. (c) Nuclear extract of the TMZ/BG/MGMT- subline was assayed for the ability to repair G•T mismatches as described in Materials and methods. Nuclear extracts of the MMR-proficient 293T Lα^+ and MMR-deficient 293T Lα^- cell lines were used as positive and negative controls, respectively. The generation of the restriction fragments of 1516 and 1307 bp is indicative of G•T to A•T repair. (d) Sixty μg of whole cell extract obtained from M14 F1 parental clone and the derived TMZ/BG/MGMT- subline were subjected to electrophoresis on a 7% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with antibodies against hMSH2, hMSH3, hMSH6, hMLH1 or hPSM2. Incubation with anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. MMR protein expression of a TMZ/01 subline derived from M14 F1 clone is also shown.

cells no demethylation of the *MGMT* promoter was observed (data not shown). We therefore decided to evaluate TMZ sensitivity of the TMZ/BG/MGMT- subline. The cells displayed a high level of TMZ resistance (the mean TMZ IC_{50} value, calculated on four independent experiments, was 324±10 μmol/l) and, as expected, TMZ resistance was unaffected by BG.

In cells devoid of MGMT activity, resistance to O^6-G-methylating agents is usually associated with a defective MMR system. We therefore evaluated MMR activity and protein expression in the TMZ/BG/MGMT- subline. The results illustrated in Fig. 6c, show that the subline was unable to repair G•T mismatches. However, the expression of all the MMR proteins was comparable between the subline and the parental clone (Fig. 6d).

Discussion

In the clinical setting, initially responsive melanomas rapidly develop resistance to TMZ (1). Drug-induced selection of resistant cell clones, pre-existing within the tumor population, may account for this phenomenon. However, unresponsiveness to TMZ could also develop as a result of drug-induced genetic and/or epigenetic alterations of neoplastic cells.

Our studies demonstrate that upon treatment with a single cycle of TMZ, mimicking patient exposure to the drug, MMR-proficient melanoma cell clones endowed with low or no MGMT activity gives rise to melanoma sublines (i.e. TMZ/01 sublines) highly resistant to the agent. As drug-induced selection of pre-existing resistant cells is unlikely to occur in clonal populations, and no changes in TMZ sensitivity were detected in the DMSO/01 sublines with respect to the parental clones, it is reasonable to hypothesize that the increased TMZ resistance of the TMZ/01 sublines is dependent on molecular alterations induced by the drug in the original clonal populations.

Up-regulation of MGMT activity appears to be one of the molecular changes underlying the increased drug resistance of the TMZ/01 sublines. Indeed, differently from their parental clones, these sublines possess high MGMT activity. Moreover, they show a significant reduction of their TMZ IC_{50} values when exposed to BG during the chemosensitivity assay. However, the finding that BG only partially reverses the acquired TMZ resistance of the TMZ/01 sublines indicates that in these sublines additional molecular changes confer protection against TMZ.

Previous studies have shown that several mutants of the human MGMT produced by site-directed or random mutagenesis retain the ability to repair O^6-MeG but become resistant to BG (5,7). Moreover, expression of some of these mutants into mammalian cells provides protection against cytotoxicity exerted by O^6-G-alkylating agents combined with BG (35-37). The reduced sensitivity of the TMZ/01 sublines to TMZ combined with BG could be due to drug-induced mutations in the *MGMT* gene, leading to the expression of a protein more resistant to BG inactivation. However, when these sublines are cultured in the presence of 5 μmol/l BG, no MGMT activity is detectable in the cells 2 h after BG exposure and after 5 days of culture with the inhibitor (data not shown). Therefore, under the experimental conditions of the chemosensitivity assay, BG is still able to abrogate MGMT activity before and during the exposure of the TMZ/01 sublines to TMZ. This finding strongly suggests that molecular changes other than MGMT mutations play a role in the reduced sensitivity of the TMZ/01 sublines to TMZ combined with BG.

Although high MGMT activity confers resistance to O^6-G-methylating agents through detoxifying the DNA, the actual killing process requires a functional MMR system. Therefore, in the absence of MGMT activity, cell sensitivity to O^6-G-methylating agents is largely, although not exclusively, dictated by MMR efficiency (21,34,38). The TMZ/01 sublines generated in our study clearly retain MMR activity, as indicated by the ability of BG to increase their sensitivity to TMZ. However, MMR could occur with a reduced efficiency. With respect to the corresponding parental clone, the GL-Mel B3/TMZ/01 subline shows a reduction of hMSH6 protein expression. This could explain, at least in part, the increased resistance of the subline to TMZ+BG. On the other hand, in the sublines established from LCP-Mel E6 and M14 F1

clones no substantial change of MMR protein expression is observed. Nevertheless, it is possible that mutations in MMR genes have occurred in these sublines, impairing the function of the corresponding proteins. In this regard, acquired defects in MMR have been previously described in Burkitt's lymphoma Raji cells exposed to *N*-methyl-*N*-nitrosourea (39).

Finally, it can not be excluded that the higher resistance to TMZ+BG displayed by the TMZ/01 sublines with respect to the parental clones may also depend on drug-induced alterations in apoptotic pathways, as previously described in melanoma cells exposed to etoposide or cisplatin (40).

An interesting result of our study is that the TMZ/04 sublines display a level of resistance to TMZ or TMZ+BG comparable to that observed in the matched TMZ/01 sublines. In this regard, it is important to note that the first cycle of TMZ treatment was performed on clonal populations, while the subsequent cycles of treatment were carried out on drug-modified heterogeneous populations. Our finding indicates that tumor cells surviving the first cycle of TMZ treatment have already become fully resistant to the drug concentration used to generate the TMZ sublines. Therefore, these cells are no longer susceptible to drug-induced selection, even though they probably continue to accumulate molecular alterations as a consequence of exposure to TMZ. In fact, previous studies performed in our laboratory revealed that mutation-dependent antigenic changes produced by triazene compounds can occur in target leukemia cells that have been rendered entirely resistant to the cytotoxic effects of these compounds (41).

In melanoma patients responding to TMZ treatment, response duration is usually disappointing. It is reasonable to hypothesize that the rapid acquisition of resistance to TMZ that we observed *in vitro* may also occur in melanoma patients exposed to the drug and substantially contribute to the early recurrence of a tumor refractory to further treatment.

In this report we addressed the question of whether the exposure of melanoma cells to BG prior to and during TMZ treatment affects the development of resistance to the methylating agent.

Our data indicate that matched TMZ and TMZ/BG sublines, generated after one or four cycles of treatment, possess comparable levels of resistance to TMZ alone. Moreover, the TMZ/BG/01 sublines, similarly to the TMZ/01 ones, display a marked increase of MGMT activity with respect to the parental clones. When BG is added to TMZ in the chemosensitivity assay, matched TMZ/01 and TMZ/BG/01 cells show comparable attenuated resistance. On the other hand, two out of three TMZ/BG/04 sublines are more resistant to TMZ+BG than matched TMZ/04 sublines. In combination, these data indicate that the overall level of TMZ resistance induced in melanoma cells is not affected by co-treatment with BG. However, cell exposure to repeated cycles of TMZ+BG appears to favor the emergence of TMZ-resistant populations in which drug resistance is much more dependent on molecular alterations not involving MGMT up-regulation.

In the present study we generated 21 TMZ/01 and 21 TMZ/BG/01 sublines from three different melanoma cell clones. Only in the TMZ/BG/01 subline group we were able to identify a subline which did not up-regulate MGMT activity. Notably, this subline is highly resistant to TMZ as a consequence of an acquired defect in MMR. Although the molecular alterations responsible for impaired MMR in this subline remain to be defined, our finding further supports the hypothesis that treatment with TMZ+BG promotes the development of MGMT-independent mechanisms of resistance to TMZ, and that an altered function of MMR may represent one of these mechanisms.

Previous investigations have shown that, with a few exceptions, MGMT activity is dictated by the efficiency of *MGMT* gene transcription (3,4). Although the molecular mechanisms involved in the regulation of *MGMT* gene transcription are complex and only partially understood, it has been demonstrated that methylation of discrete regions within the *MGMT* promoter is associated with the silencing of the gene, whereas methylation of the body of the gene directly correlates with the level of its transcription (3,4).

Our results demonstrate that up-regulation of MGMT activity in TMZ/01 and TMZ/BG/01 sublines is associated with changes in the methylation status of either the promoter or the body of the *MGMT* gene. Moreover, consistent with previous studies, our data illustrate that although an unmethylated promoter is required for *MGMT* expression the actual levels of expression are directly correlated with the levels of methylation in the body of the gene. Indeed, in LCP-Mel E6 and GL-Mel B3 parental clones, which are endowed with detectable but very low MGMT activity, the *MGMT* promoter region analyzed is completely unmethylated, while the body region displays only few methylated CpG sites. On the other hand, in the TMZ/01 and TMZ/BG/01 sublines derived from these two clones and expressing high MGMT activity, the promoter is still unmethylated, but nearly all the CpG sites within the body of the gene are methylated. Moreover, exposure of the LCP-Mel E6 TMZ/01 subline to 5-AZA reduces both MGMT activity and methylation in the body of the *MGMT* gene.

In M14 F1 clone, which is devoid of MGMT activity, the *MGMT* promoter is fully methylated and the body region hypermethylated. Increased MGMT activity in the TMZ/01 and TMZ/BG/01 sublines derived from this clone is associated with a partial demethylation of the promoter and a slight decrease in the level of methylation of the body of the *MGMT* gene. However, in the M14 F1 TMZ/01 subline both MGMT activity and methylation of the *MGMT* body are reduced upon treatment with 5-AZA. This finding strongly suggests that the up-regulation of MGMT activity observed in the M14 F1 TMZ/01 and TMZ/BG/01 sublines could be dependent on demethylation of the *MGMT* gene promoter.

Increased MGMT activity due to hypermethylation of the body of the *MGMT* gene has been previously reported in melanoma cells with acquired resistance to fotemustine (42). Reactivation of a silent *MGMT* gene has also been demonstrated by Bearzatto *et al* (43) in MGMT-deficient Raji cells surviving treatment with *N*-methyl-*N*-nitrosourea. In this latter study, hypermethylation of an *MGMT* region outside the promoter and the first exon was suggested to be involved in gene reactivation. Our results are consistent with these findings and demonstrate that, depending on the initial methylation pattern of the *MGMT* gene in cells, drug-induced up-regulation of MGMT activity can occur either through a demethylation of the gene promoter or hypermethylation of the body of the gene.

In conclusion, this study demonstrates that a single cycle of *in vitro* treatment with TMZ designed to mimic patient exposure to the drug is sufficient to induce a marked increase of resistance to the agent in clonal melanoma cell populations. Up-regulation of MGMT activity, as well as additional molecular changes, including impaired MMR, account for the acquired TMZ resistance. Melanoma cells exposed to TMZ+BG acquire a level of TMZ resistance comparable to that of the cells exposed to TMZ alone. However, combined treatment of TMZ with BG appears to promote the development of MGMT-independent mechanisms of TMZ resistance. Although further studies will be required to identify the MMR- and MGMT-independent mechanisms of TMZ resistance, the biochemical events revealed by the present investigation provide molecular bases to understand, at least in part, the rapid onset of drug resistance that is constantly seen in the triazene-based chemotherapy of melanoma patients.

Our data also illustrate that TMZ-induced up-regulation of MGMT activity can result from *MGMT* promoter demethylation or hypermethylation of the body of the gene, depending on the initial *MGMT* methylation pattern in the cells. In both conditions, TMZ-induced up-regulation of MGMT activity can be, at least in part, reversed by 5-AZA treatment. Therefore, 5-AZA could be a reasonably good candidate to play a role in limiting the extent of acquired resistance of melanoma cells in patients exposed to triazene compounds.

Acknowledgements

The authors thank Maurizio Inzillo for the artwork. This work was supported in part by Italian Ministry of Education, University and Research grant FIRB-RBNE01LFNY-006 to S.D., and in part by Italian Ministry of Health grant RF03-200 to S.D.

References

1. Li Y and McClay EF: Systemic chemotherapy for the treatment of metastatic melanoma. Semin Oncol 29: 413-426, 2002.
2. Stevens MF, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig G, Goddard C, Gibson NW, Slack JA, *et al*: Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res 47: 5846-5852, 1987.
3. Pegg AE, Dolan ME and Moschel RC: Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51: 167-223, 1995.
4. Margison GP, Povey AC, Kaina B and Santibanez Koref MF: Variability and regulation of O^6-alkylguanine-DNA alkyltransferase. Carcinogenesis 24: 625-635, 2003.
5. Dolan ME and Pegg AE: O^6-benzylguanine and its role in chemotherapy. Clin Cancer Res 3: 837-847, 1997.
6. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT and Brock C: Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev 23: 35-61, 1997.
7. Gerson SL: Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 20: 2388-2399, 2002.
8. Stojic L, Brun R and Jiricny J: Mismatch repair and DNA damage signalling. DNA Repair 3: 1091-1101, 2004.
9. Karran P: Mechanisms of tolerance to DNA damaging therapeutic drugs. Carcinogenesis 22: 1931-1937, 2001.
10. Kaina B, Ziouta A, Ochs K and Coquerelle T: Chromosomal instability, reproductive cell death and apoptosis induced by O^6-methylguanine in Mex^-, Mex^+ and methylation-tolerant mismatch repair compromised cells: facts and models. Mutat Res 381: 227-241, 1997.
11. Bignami M, O'Driscoll M, Aquilina G and Karran P: Unmasking a killer: DNA O^6-methylguanine and the cytotoxicity of methylating agents. Mutat Res 462: 71-82, 2000.
12. D'Atri S, Tentori L, Lacal PM, Graziani G, Pagani E, Benincasa E, Zambruno G, Bonmassar E and Jiricny J: Involvement of the mismatch repair system in temozolomide-induced apoptosis. Mol Pharmacol 54: 334-341, 1998.
13. Hirose Y, Katayama M, Stokoe D, Haas-Kogan DA, Berger MS and Pieper RO: The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G_2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. Mol Cell Biol 23: 8306-8315, 2003.
14. Stojic L, Mojas N, Cejka P, Di Pietro M, Ferrari S, Marra G and Jiricny J: Mismatch repair-dependent G_2 checkpoint induced by low doses of SN1 type methylating agents requires the ATR kinase. Genes Dev 18: 1331-1344, 2004.
15. Caporali S, Falcinelli S, Starace G, Russo MT, Bonmassar E, Jiricny J and D'Atri S: DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. Mol Pharmacol 66: 478-491, 2004.
16. Hirose Y, Berger MS and Pieper RO: p53 affects both the duration of G_2/M arrest and the fate of temozolomide-treated human glioblastoma cells. Cancer Res 61: 1957-1963, 2001.
17. Fishel R: Signaling mismatch repair in cancer. Nat Med 5: 1239-1241, 1999.
18. Fink D, Aebi S and Howell SB: The role of DNA mismatch repair in drug resistance. Clin Cancer Res 4: 1-6, 1998.
19. Wedge SR, Porteous JK and Newlands ES: 3-aminobenzamide and/or O^6-benzylguanine evaluated as an adjuvant to temozolomide or BCNU treatment in cell lines of variable mismatch repair status and O^6-alkylguanine-DNA alkyltransferase activity. Br J Cancer 74: 1030-1036, 1996.
20. Wedge SR, Porteous JK and Newlands ES: Effect of single and multiple administration of an O^6-benzylguanine/temozolomide combination: an evaluation in a human melanoma xenograft model. Cancer Chemother Pharmacol 40: 266-272, 1997.
21. Pepponi R, Marra G, Fuggetta MP, Falcinelli S, Pagani E, Bonmassar E, Jiricny J and D'Atri S: The effect of O^6-alkylguanine-DNA alkyltransferase and mismatch repair activities on the sensitivity of human melanoma cells to temozolomide, 1,3-bis(2-Chloroethyl)1-nitrosourea, and cisplatin. J Pharmacol Exp Ther 304: 661-668, 2003.
22. Golub SH, Hanson DC, Sulit HL, Morton DL, Pellegrino MA and Ferrone S: Comparison of histocompatibility antigens on cultured human tumor cells and fibroblasts by quantitative antibody absorption and sensitivity to cell-mediated cytotoxicity. J Natl Cancer Inst 56: 167-170, 1976.
23. Goldmacher VS, Cuzick RAJ and Thilly WG: Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine. J Biol Chem 261: 12462-12471, 1986.
24. Aquilina G, Ceccotti S, Martinelli S, Hampson R and Bignami M: N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea sensitivity in mismatch repair-defective human cells. Cancer Res 58: 135-141, 1998.
25. Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG: Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59: 793-797, 1999.
26. Cejka P, Stojic L, Mojas N, Russell AM, Heinimann K, Cannavò E, Di Pietro M, Marra G and Jiricny J: Methylation-induced G_2/M arrest requires a full complement of the mismatch repair protein hMLH1. EMBO J 22: 2245-2254, 2003.
27. Hammond LA, Eckardt JR, Baker SD, Eckhardt SG, Dugan M, Forral K, Reidenberg P, Statkevich P, Weiss GR, Rinaldi DA, von Hoff DD and Rowinsky EK: Phase I and pharmacokinetic study of temozolomide on a daily-for-5-days schedule in patients with advanced solid malignancies. J Clin Oncol 17: 2604-2613, 1999.
28. Holmes JJ, Clark S and Modrich P: Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. Proc Natl Acad Sci USA 87: 5837-5841, 1990.
29. Lacal PM, D'Atri S, Orlando L, Bonmassar E and Graziani G: *In vitro* inactivation of human O^6-alkylguanine DNA alkyltransferase by antitumor triazene compounds. J Pharmacol Exp Ther 279: 416-422, 1996.

30. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
31. Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS and Futscher BW: Methylation of discrete regions of the O^6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. Mol Cell Biol 17: 5612-5619, 1997.
32. Esteller M, Catasus L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB and Herman JG: hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 155: 1767-1772, 1999.
33. Xiong Z and Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25: 2532-2534, 1997.
34. Dosch J, Christmann M and Kaina B: Mismatch G-T binding activity and MSH2 expression is quantitatively related to sensitivity of cells to methylating agents. Carcinogenesis 19: 567-573, 1998.
35. Loktionova NA and Pegg AE: Point mutations in human O^6-alkylguanine-DNA alkyltransferase prevent the sensitization by O^6-benzylguanine to killing by N,N'-bis (2-chloroethyl)-N-nitrosourea. Cancer Res 56: 1578-1583, 1996.
36. Christians FC, Dawson BJ, Coates MM and Loeb LA: Creation of human alkyltransferases resistant to O^6-benzylguanine. Cancer Res 57: 2007-2012, 1997.
37. Davis BM, Encell LP, Zielske SP, Christians FC, Liu L, Friebert SE, Loeb LA and Gerson SL: Applied molecular evolution of O^6-benzylguanine-resistant DNA alkyltransferases in human hematopoietic cells. Proc Natl Acad Sci USA 98: 4950-4954, 2001.
38. Marra G, D'Atri S, Corti C, Bonmassar L, Cattaruzza MS, Schweizer P, Heinimann K, Bartosova Z, Nystrom-Lahti M and Jiricny J: Tolerance of human $MSH2^{+/-}$ lymphoblastoid cells to the methylating agent temozolomide. Proc Natl Acad Sci USA 98: 7164-7169, 2001.
39. Hampson R, Humbert O, Macpherson P, Aquilina G and Karran P: Mismatch repair defects and O^6-methylguanine-DNA methyltransferase expression in acquired resistance to methylating agents in human cells. J Biol Chem 272: 28596-28606, 1997.
40. Helmbach H, Kern MA, Rossmann E, Renz K, Kissel C, Gschwendt B and Schadendorf D: Drug resistance towards etoposide and cisplatin in human melanoma cells is associated with drug-dependent apoptosis deficiency. J Invest Dermatol 118: 923-932, 2002.
41. Fioretti MC, Bianchi R, Romani L and Bonmassar E: Drug-induced immunogenic changes of murine leukemia cells: dissociation of onset of resistance and emergence of novel immunogenicity. J Natl Cancer Inst 71: 1247-1251, 1983.
42. Christmann M, Pick M, Lage H, Schadendorf D and Kaina B: Acquired resistance of melanoma cells to the antineoplastic agent fotemustine is caused by reactivation of the DNA repair gene MGMT. Int J Cancer 92: 123-129, 2001.
43. Bearzatto A, Szadkowski M, Macpherson P, Jiricny J and Karran P: Epigenetic regulation of the MGMT and hMSH6 DNA repair genes in cells resistant to methylating agents. Cancer Res 60: 3262-3270, 2000.