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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

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ABSTRACT

The prognosis of advanced melanoma is generally poor, because this tumor commonly exhibits intrinsic or acquired resistance to chemotherapy. In an attempt to identify the underlying causes of this resistance, we studied the roles played by the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (OGAT) and the mismatch repair (MMR) system in the sensitivity of melanoma cells to temozolomide (TMZ), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or *cis*-diamminedichloroplatinum(II) (CDDP). To this end, OGAT levels and MMR efficiency of extracts of nine melanoma cell lines and selected clones derived from four of these lines were determined and correlated with the sensitivity of the respective cells to these drugs. The effectiveness of O^6-benzylguanine (BG), a specific OGAT inhibitor, in potentiating TMZ- or BCNU-mediated cytotoxicity was also evaluated. Our results demonstrate that MMR efficiency and OGAT levels strongly affect melanoma cell sensitivity to TMZ. In MMR-proficient cells, a direct correlation between OGAT levels and TMZ IC_{50} values was found. When OGAT activity was inhibited with BG, the sensitivity of these cells to TMZ increased and was then dictated largely by their MMR efficiency. MMR-deficient cells were highly resistant to the drug irrespective of their OGAT levels. Although OGAT activity and MMR status seemed to be the major determinants of melanoma sensitivity to TMZ, this was not the case for BCNU and CDDP; resistance to the latter drugs clearly involves processes other than the two DNA repair pathways analyzed in this study.

Management of disseminated melanoma represents one of the most challenging problems in clinical oncology, because both single-agent and combination therapies give disappointing results in the treatment of this form of neoplasia (for review, see Huncharek et al., 2001). Chemoresistance observed in vivo is reflected in vitro by low chemosensitivity of melanoma cell lines (Schadendorf et al., 1994), suggesting the presence of intrinsic cellular resistance mechanisms. Some of these might involve inhibition or dysregulation of apoptosis (for review, see Serrone and Hersey, 1999; Helmbach et al., 2001, 2002),whereas others might be linked to the levels of DNA repair enzymes that either attenuate or potentiate the effect of the drugs.

One of the key factors controlling sensitivity to clinical alkylating agents that attack the O^6 position of guanine is the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (OGAT) (for review, see Pegg, 1990; Pegg et al., 1995; Gerson, 2002). This detoxifying protein removes small alkyl groups from O^6-guanine in DNA and transfers them to an internal cysteine residue in a stoichiometric and autoinactivating reaction. Thus, tumor cells with high OGAT levels are generally more resistant to temozolomide or 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (TMZ), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) than OGAT-deficient cell lines (Pegg, 1990; Pegg et al., 1995; Gerson, 2002). Correspondingly, depletion of OGAT activity by the competitive inhibitor O^6-benzylguanine (BG; for review, see Dolan and Pegg, 1997) increases tumor cell

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ABBREVIATIONS: OGAT, O^6-alkylguanine-DNA alkyltransferase; TMZ, temozolomide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; BG, O^6-benzylguanine; MMR, mismatch repair; CDDP, *cis*-diamminedichloroplatinum(II); O^6-MeG, O^6-methylguanine; CM, complete medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; STE, short-term exposure; LTE, long-term exposure.

sensitivity to triazene compounds and chloroethylnitrosoureas both in vitro and in vivo (Pegg, 1990; Pegg et al., 1995; Dolan and Pegg, 1997; Gerson, 2002).

Experimental evidence obtained during the past few years has implicated the mismatch repair (MMR) system in the cellular response to chemotherapeutic agents. Loss of MMR is associated with resistance to TMZ, 6-thioguanine, *cis*-diaminedichloroplatinum(II) (CDDP), doxorubicin, and etoposide (for review, see Fink et al., 1998), and with hypersensitivity to CCNU (Aquilina et al., 1998). The MMR system, dedicated to the correction of biosynthetic errors (for review, see Modrich, 1997; Jiricny and Nyström-Lahti, 2000), involves the hMSH2, hMSH3, hMSH6, hMLH1, and hPMS2 polypeptides, as well as other proteins that participate in DNA metabolism (Modrich, 1997; Jiricny and Nyström-Lahti, 2000). When cells treated with methylating agents undergo DNA replication, unrepaired O^6-methylguanine (O^6-MeG) residues in the template strand form mismatches with thymines, which serve as substrates for the MMR system. However, because the repair process is directed to the newly synthesized strand and because no better match other than thymine can be found for O^6-MeG, reiterated futile attempts of the MMR system to correct the O^6-MeG/T mismatches might lead to cell death (for review, see Karran, 2001). In cells treated with CDDP, the MMR system may promote cell death by preventing recombinational repair of double-strand breaks that arise as secondary lesions of drug-induced DNA modifications (Karran, 2001). In contrast, in cells exposed to CCNU, it has been hypothesized that MMR participates in the removal of the interstrand cross-links generated by unrepaired O^6-(2-chloroethyl)guanine, thus providing protection against cytotoxicity of the drug (Aquilina et al., 1998).

BCNU and CDDP are commonly used in the treatment of melanoma (Huncharek et al., 2001), TMZ is in phase III clinical trials (Middleton at al., 2000), and BG is in phase I/II clinical studies to assess the effect of OGAT inhibition on tumor cells resistant to TMZ or BCNU (Gerson, 2002). However, the relationship between OGAT levels and TMZ or BCNU sensitivity, as well as the therapeutic potential of BG as inhibitor of the enzyme, have to date been investigated in only two melanoma cell lines (Dolan et al., 1991; Wedge et al., 1996a,b) and in a human melanoma xenograft model (Wedge et al., 1997). Moreover, no studies examined the effect of MMR status on the cytotoxicity of TMZ, BCNU, and CDDP in melanoma cells. We set out to readdress this situation by evaluating the influence of OGAT and MMR activities on melanoma cell sensitivity to TMZ, BCNU, and CDDP and on the ability of BG to potentiate the cytotoxic effects of the former two drugs. To this end, we used several MMR-proficient melanoma cell lines and clones, as well as the recently established MMR-deficient human melanoma cell lines PR-Mel, which does not express the hMLH1 and hPMS2 proteins (Alvino et al., 2002), and PD-Mel, which lacks hMSH6. We show that in melanoma cells resistance to TMZ is controlled principally by the OGAT and MMR systems, whereas resistance to BCNU and CDDP involves other factors as well.

Materials and Methods

Cell Lines and Clones. Nine human melanoma cell lines were used in this study. M14 and M10 (Golub et al., 1976) were kindly provided by Dr. G. Zupi (Istituto Regina Elena, Rome, Italy); LCP-MEL, GL-MEL, and PD-Mel (Lacal et al., 2000) were kindly provided by Dr. Guadagni (Istituto Regina Elena); CR-Mel, PR-Mel, and CN-Mel (Alvino et al., 2002) were established in our laboratory; and SK-Mel-28 was obtained from the American Type Culture Collection (Manassas, VA). LCP-Mel was obtained from a primary melanoma; PR-Mel, M14, and M10 were established from cutaneous metastases; and the remaining cell lines were originated from noncutaneous metastases, including lymph node and organ metastases.

Clonal populations were obtained from PR-Mel, M14, LCP-MEL, and GL-MEL lines by limiting dilution. All cell lines and clones were cultured at 37°C in 5% CO_2-humidified atmosphere and maintained in RPMI 1640 medium (Hyclone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and antibiotics (Invitrogen, Paisley, Scotland) (referred to as complete medium, CM).

We have previously shown that the PR-Mel cells are MMR-deficient, whereas CR-Mel and CN-Mel cells are MMR-proficient (Alvino et al., 2002). All the other cell lines were characterized for MMR activity in the present study.

Drugs and Reagents. TMZ was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). BG and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). CDDP (Prontoplatamine, 500 μg/ml in saline, pH 3–5) was purchased from Pharmacia & Upjhon (Milan, Italy). BCNU (Nitrumon) was obtained from Sintesa S.A (Brussels, Belgium).

BG was dissolved in ethanol (2.4 mg/ml), stored as stock solution at −80°C, and diluted in CM just before use. BCNU solution was prepared freshly and discarded after use. The drug was dissolved in ethanol (50 mg/ml) and then diluted in CM. The final concentrations of ethanol in the cultures treated with BG and/or BCNU did not affect cell growth (data not shown). TMZ was also prepared freshly in CM (388 μg/ml) and discarded after use. MTT was prepared at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) and stored at 4°C.

Reagents for SDS-polyacrylamide gel electrophoresis were all purchased from Bio-Rad (Hercules, CA).

Monoclonal Antibodies and Western Blot Analysis. 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; and mAb against actin (clone AC-40) was from Sigma-Aldrich.

Western blot analysis of MMR protein expression was performed as described previously (Alvino et al., 2002). The mAb against actin was used as an internal standard for protein loading.

Mismatch Repair Assay. The efficiency of protein extracts in repairing DNA mismatches was tested as described previously (Thomas et al., 1991). Briefly, M13 mp2 heteroduplexes containing a G/T mismatch were incubated with protein extracts for 20 min at 37°C. The DNA was then purified, electroporated into a *mutS* strain of *Escherichia coli*, and plated along with the α-complementation strain CSH50, isopropyl β-D-thiogalactoside, and 5-bromo-4-chloro-3-indolyl β-D-galactoside. If no repair occurred, a high percentage of mixed plaques, containing both blue and colorless progeny, was observed. Reduction in the percentage of mixed plaques and a concomitant increase in single-color plaques were indicative of repair. In the complementation studies, the assays were carried out as described above, except that the extracts (50 μg) were supplemented with purified recombinant hMutSα or hMutLα (0.1 μg each). Repair efficiency of cell extracts was calculated according to the following

formula: % of repair = 100 × [1 − (% of mixed plaques in extract-treated samples)/(% of mixed plaques in extract-untreated samples). Cell lines with repair efficiency lower that 20% were considered MMR-deficient.

OGAT Assay. Melanoma cells, either untreated or exposed to BG, were removed from continuous culture, washed twice with PBS, and stored as pellets at −80°C until used. OGAT activity was determined by measuring the transfer of ^{3}H-methyl groups from a DNA substrate to the OGAT protein (Watson and Margison, 1999). Briefly, cell pellets (1×10^6 cells) were resuspended in 1 ml of lysis buffer (0.5% CHAPS, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, and 10% glycerol) supplemented with a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany) and incubated 30 min at 4°C. Cell lysates were then centrifuged at 18,000*g* for 10 min at 4°C. 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 ^{3}H-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 was precipitated using 1 mg of bovine serum albumin as carrier. Pellets were washed with 1 N perchloric acid, resuspended in 0.01 N NaOH, and radioactivity measured in a liquid scintillation counter (Tri-Carb 1900; Packard BioScience, CT), after addition of scintillation liquid (Ultima Gold; Packard Instruments Chemical Operation, Groningen, The Netherlands). Protein concentration in supernatants was evaluated according to the method of Bradford using the Bio-Rad dye solution and bovine serum albumin as standard. OGAT activity was expressed in terms of femtomoles of ^{3}H-methyl groups transferred per milligram of protein in cell extract.

Drug Treatment and Evaluation of Cell Chemosensitivity by the MTT Assay. Melanoma cells were suspended in CM at a concentration of 2×10^4 cells/ml, dispensed in 50-μl aliquots into flat-bottom 96-well plates (Falcon; BD Biosciences, Franklin Lakes, NJ), and allowed to adhere overnight. Graded amounts of each drug 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 5 days. Four replica wells were used for controls and each drug concentration. TMZ was tested at concentrations ranging between 7.81 and 1000 μM, CDDP at concentrations ranging between 0.78 and 50 μM, and BCNU at concentrations ranging between 3.12 and 200 μM.

The cytotoxic effects of TMZ and BCNU were evaluated also in combination with the OGAT inhibitor BG to prevent repair of the methyl adducts at the O^6-G. In the case of TMZ, two different schedules of BG treatment were used: 1) short-term exposure (STE) to BG, performed by treating cells with 5 μM BG for 2 h and removing the inhibitor before drug exposure and 2) long-term exposure (LTE) to BG, carried out by treating cells with 5 μM BG 2 h before and during drug exposure. The LTE but not the STE schedule was adopted when tumor cells were subjected to BCNU + BG treatment. Control groups were either untreated or treated with BG alone.

The MTT assay was performed as described previously (Hansen et al., 1989). Briefly, after 5 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 an 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. Correlation coefficient was obtained using simple regression analysis (Excel software). *P* values were calculated according to Student's *t* test analysis.

Results

OGAT Activity in Melanoma Cell Lines and Clones. We first determined the levels of OGAT in the nine melanoma cell lines. The results illustrated in Fig. 1A and in Table 1 show that OGAT activity in the bulk lines ranged from undetectable in CR-Mel cells to 827 fmol/mg of protein in the CN-Mel line.

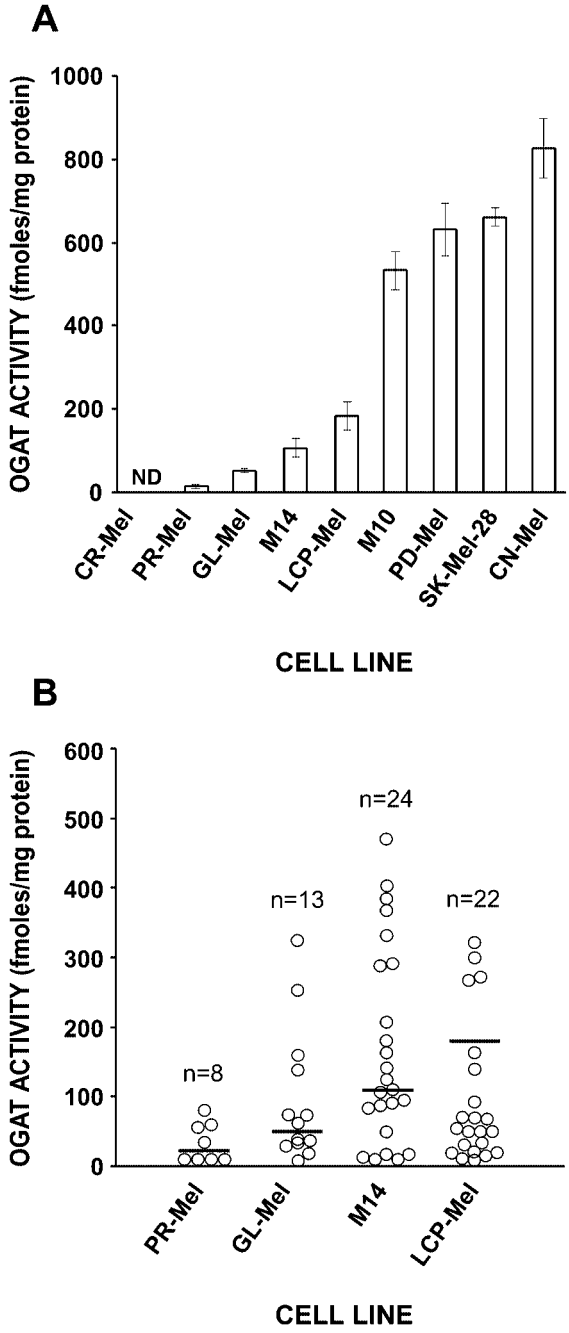

Fig. 1. OGAT activity in human melanoma cell lines and clones. A, bulk populations of nine melanoma cell lines were tested for OGAT activity, as described under *Materials and Methods*. The enzyme activity was expressed in terms of femtomoles of methyl groups removed per milligram of protein in cell extracts. Each value represents the arithmetic means of at least three independent experiments. Bars, standard error of the mean. ND, not detectable. B, PR-Mel, GL-Mel, M14, and LCP-Mel were cloned by limiting dilution. For each cell line, the indicated number of clones was tested for OGAT activity. Horizontal bars represent OGAT levels detected in the bulk population.

TABLE 1
OGAT activity, MMR efficiency, and sensitivity to TMZ, BCNU, or CDDP of human melanoma cell lines

Cell Line[a]	OGAT[b]	% MMR[c]	IC_{50}[d]				
			TMZ	TMZ + LTE/BG[e]	BCNU	BCNU + LTE/BG[e]	CDDP
					μM		
CR-Mel	N.D.	35–39	92 ± 16	103 ± 13	38 ± 5.4	40 ± 7.4	4.0 ± 0.6
LCP-Mel-Clone E4	N.D.	44–46	103 ± 14	103 ± 8.0	13 ± 1.1	12 ± 1.2	8.5 ± 0.3
PR-Mel	12 ± 6	3–11	>1000	>1000	17 ± 1.5	17 ± 0.6	7.0 ± 0.9
GL-Mel	52 ± 6	77–88	148 ± 17	36 ± 6.5**	47 ± 2.5	37 ± 3.4*	8.7 ± 0.9
M14	107 ± 21	87–93	101 ± 11	23 ± 3.6**	82 ± 10	55 ± 6.0*	11 ± 1.2
LCP-Mel	184 ± 33	37–40	218 ± 20	143 ± 32*	13 ± 1.0	8.2 ± 0.5**	5.6 ± 0.3
LCP-Mel-Clone E3	331 ± 46	45–47	368 ± 36	145 ± 35**	45 ± 4.4	9.2 ± 1.3**	8.3 ± 1.2
M10	533 ± 45	89–92	301 ± 28	24 ± 4.0**	57 ± 5.5	22 ± 2.4**	3.3 ± 0.3
PD-Mel	631 ± 64	2–9	448 ± 32	436 ± 43	47 ± 3.5	22 ± 1.4**	19 ± 2.5
SK-Mel-28	660 ± 22	43–49	688 ± 47	331 ± 13**	70 ± 2.5	34 ± 0.3**	11 ± 0.9
CN-Mel	827 ± 72	90–94	778 ± 32	369 ± 13**	74 ± 2.9	35 ± 2.1**	20 ± 4.5

N.D., not detectable.
[a] Cells were incubated with graded concentrations of the indicated drugs for 5 days and then analyzed for cell growth by the MTT assay.
[b] OGAT activity is expressed in terms of fmoles of ^{3}H-methyl groups transferred per milligram of protein in cell extract. Each value represents the mean ± standard error of the mean of at least three independent experiments.
[c] Efficiency of MMR displayed by the cell line in two independent experiments.
[d] Drug concentration required to inhibit cell growth by 50%. Each value represents the mean ± standard error of the mean of at least three independent experiments.
[e] LTE/BG was performed by incubating cells with 5 μM BG for 2 h before treatment with TMZ or BCNU and maintaining the inhibitor in culture up to the end of the assay.
** $P < 0.01$, * $P < 0.05$ 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.

We next cloned the four cell lines expressing low (PR-Mel and GL-Mel) or moderate (LCP-Mel and M14) OGAT activity and evaluated the enzyme levels in 67 clones total. Ample variation of OGAT activity was found at clonal level, too (Fig. 1B). Notably, all four lines contained a number of clones with OGAT activities significantly higher that those of the respective bulk populations.

MMR Activity and MMR Protein Expression in Melanoma Cell Lines. PR-Mel cells have been previously shown to be MMR-deficient due to the loss of hMutLα expression, whereas CR-Mel and CN-Mel have been shown to be MMR-proficient (Alvino et al., 2002). Analysis of MMR activities of the additional six cell lines used in the present investigation showed that, with the exception of PD-Mel, all were able to repair G/T mismatches, albeit with different efficiency (Fig. 2A; Table 1). Addition of purified recombinant hMutSα, but not hMutLα, to PD-Mel extracts restored the mismatch repair activity to 90% (data not shown), indicating that PD-Mel cells harbor a defect in hMSH2 or hMSH6.

In agreement with the result of the MMR assay, expression of all MMR proteins was present in the MMR-proficient cell lines, whereas no hMSH6 was detectable in the extracts of PD-Mel cell line (Fig. 2B). The immunoblots of Fig. 2B also reveal that the amount of hMSH6 was reduced in LCP-Mel cells compared with that detectable in the other melanoma cell lines.

Relationship between OGAT Levels, MMR Activity, and Sensitivity of Melanoma Cell Lines to TMZ, BCNU, and CDDP. All nine melanoma cell lines, as well as two clones derived from LCP-Mel, were tested for sensitivity to TMZ, BCNU, and CDDP. The LCP-Mel clones were chosen for having higher or lower OGAT activities than the parental bulk line. The effects of BG on melanoma cell susceptibility to the cytotoxic effects of TMZ and BCNU were also evaluated.

The results illustrated in Table 1 and Fig. 3A show that in the MMR-proficient cells a direct correlation was detected between OGAT levels and TMZ IC_{50} values. STE to BG did not influence cell sensitivity to TMZ (data not shown), whereas LTE significantly increased the cytotoxic effects of the drug in all cell lines, with the exception of those devoid of OGAT activity (Table 1). The MMR-deficient cell lines PR-Mel and PD-Mel were highly resistant to TMZ and no increase of chemosensitivity was observed upon LTE to BG (Table 1). Figure 4A is a plot of the TMZ IC_{50} values (with LTE to BG) against the MMR efficiency of the respective cell lines. The data show that in the absence of OGAT activity, sensitivity of MMR-proficient melanoma cells to TMZ is largely dictated by the efficiency of MMR. M10, M14, and GL-Mel cell lines (MMR efficiency about 80–90%) were about 5-fold more sensitive to the drug than LCP-Mel, LCP-Mel clone E3, LCP-Mel clone E4, and CR-Mel, which displayed a MMR efficiency of about 40%. However, CN-Mel and SK-Mel-28 cell lines were less sensitive to TMZ than expected on the basis of their MMR efficiency.

In the case of BCNU, the relationship between OGAT activity and IC_{50} values was less linear than that observed with TMZ (Fig. 3B; Table 1). CR-Mel, GL-Mel, and PD-Mel, although possessing undetectable, low and high OGAT activities, respectively, were similarly sensitive to BCNU, whereas the M14 cell line, which had moderate OGAT activity, was the most resistant. Because STE to BG failed to increase the sensitivity of the melanoma cells to TMZ, only the effects of an LTE to BG were evaluated in cells treated with BCNU. This treatment schedule with BG increased sensitivity to BCNU of all melanoma cell lines, with the exception of those displaying no or barely detectable OGAT activities (Table 1). As observed for TMZ, significant differences in BCNU sensitivity were still present among the cell lines even after OGAT depletion (Table 1). Previous studies have shown that the MMR system provides protection against CCNU cytotoxicity, possibly through the participation in the repair of interstrand DNA cross-links generated by the drug (Aquilina et al., 1998). Because BCNU produces the same type of interstrand DNA cross-links as CCNU, we wanted to test whether, in the absence of OGAT activity, the efficiency of MMR was also correlated with cell sensitivity to BCNU. The results presented in Fig. 4B show that the MMR system does indeed protect the cells against killing by the drug, albeit weakly.

The bulk melanoma cell lines and the two selected clones

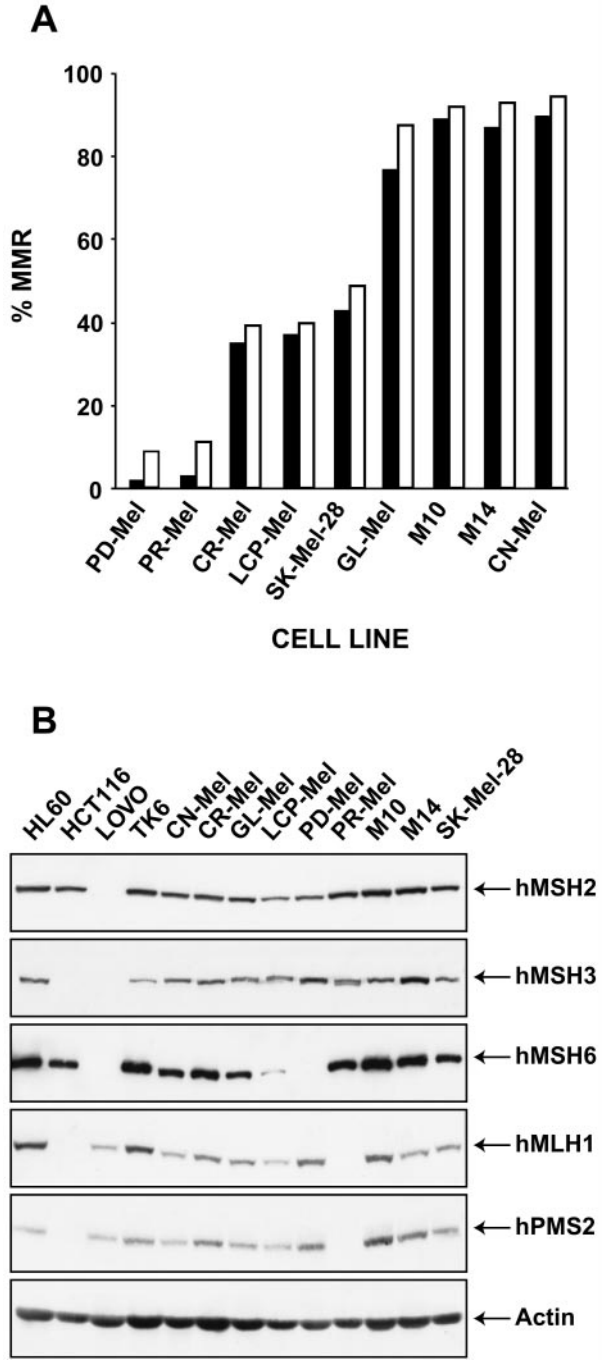

Fig. 2. MMR activity and MMR protein expression in human melanoma cell lines. A, protein extracts of melanoma cells were tested for MMR efficiency using a substrate containing a single G/T mispair (see *Materials and Methods*). For each cell line, the results of two independent experiments are shown. B, 80 μg of the whole cell extracts 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 enhanced chemiluminescence. Controls, LOVO cells, expressing hMLH1 and hPSM2 but not hMSH2 and hMSH6; HCT116 cells, expressing hMSH2 and hMSH6 but not hMLH1 and hPMS2; and MMR-proficient cell lines HL60 and TK6.

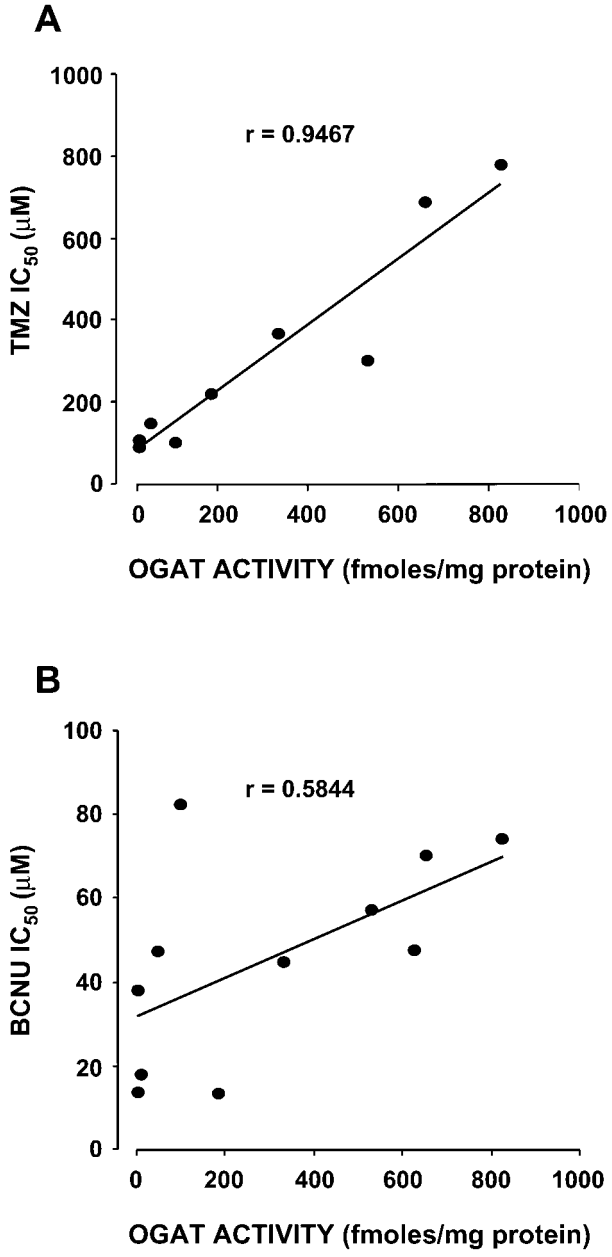

Fig. 3. Relationship between OGAT activity and sensitivity of human melanoma cell lines to TMZ or BCNU. A, for each MMR-proficient melanoma cell line, the TMZ IC_{50} value (Table 1) was plotted against OGAT activity. Correlation coefficient was obtained using simple regression analysis (Excel software). B, BCNU IC_{50} values of all the melanoma cell lines included in Table 1 were plotted against OGAT activity. The correlation coefficient was calculated as in A.

also showed different sensitivity to CDDP. However, the MMR status did not seem to correlate with resistance to the drug (Fig. 4C)

Kinetics of OGAT Recovery after BG-Mediated Depletion. Treatment with 2 μM BG for 2 h reduced OGAT activity to undetectable levels in all melanoma lines (data not shown). We therefore wanted to test whether the lack of sensitization to TMZ after STE to BG could be explained by a rapid recovery of the OGAT activity after BG removal. GL-Mel, LCP-Mel, and SK-Mel-28 cell lines were subjected to STE to BG and maintained in culture for 72 h. OGAT activity was assayed at the end of the treatment and every 24 h after BG removal. The results illustrated in Fig. 5, expressed in terms of percentage of OGAT activity in BG-treated cells with respect to controls, show that more than 70% of control

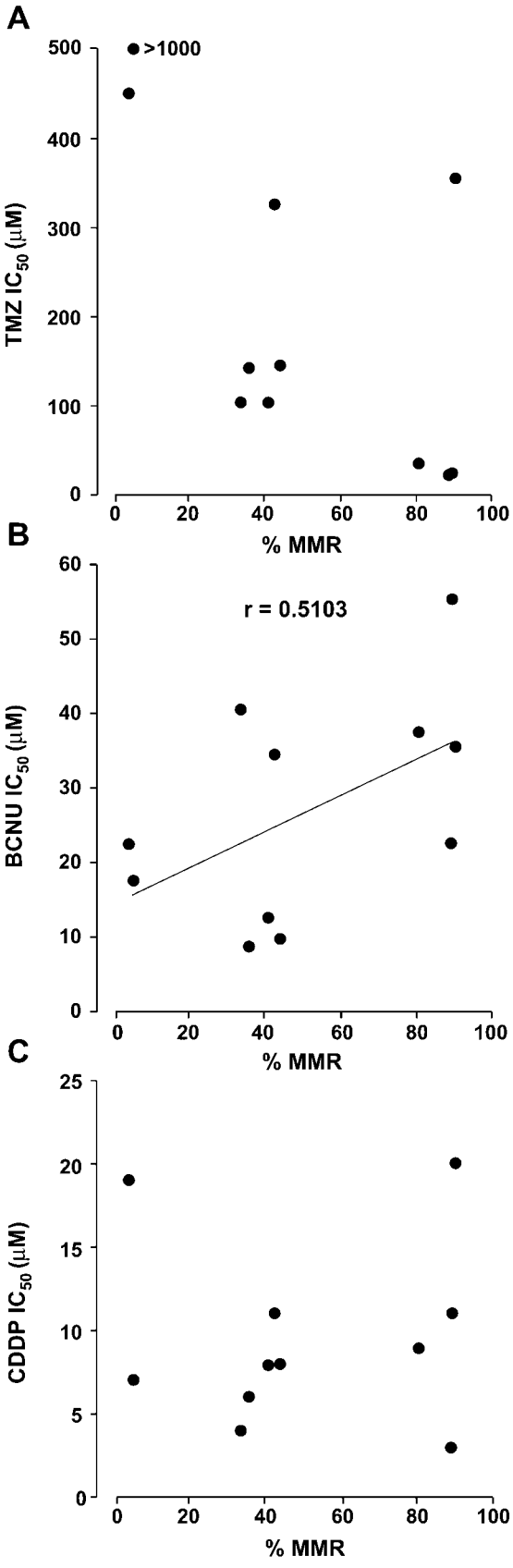

Fig. 4. Relationship between MMR activity and sensitivity of human melanoma cell lines to TMZ, BCNU, and CDDP. For each melanoma cell line the IC_{50} value of TMZ (A), BCNU (B), or CDDP (C) was plotted against MMR activity (also see Table 1).

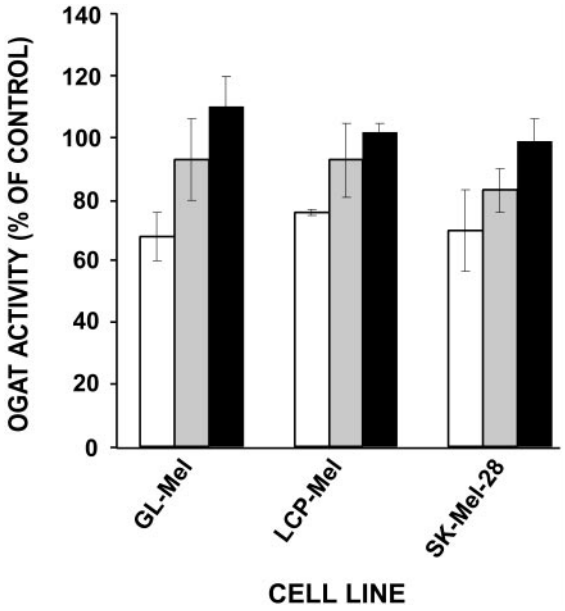

Fig. 5. Recovery of OGAT activity in melanoma cell lines subjected to STE to BG. GL-Mel, LCP-Mel, and SK-Mel-28 cells were treated with 5 μM BG for 2 h, washed, and incubated in CM for 72 h. Cell extracts were prepared and tested for OGAT activity after 24 h (□), 48 h (▨), and 72 h (■) of culture. OGAT activity of BG-treated cells was expressed as percentage of control activity, detected at the same time points. Each value represents the mean of three independent experiments. Bars, standard error of the mean.

OGAT levels were regenerated after 24 h of cell culture in the absence of BG. In contrast, in all cell lines subjected to LTE to BG, no OGAT recovery occurred up to 5 days of culture (data not shown).

Discussion

We set out to study the roles played by OGAT and by the MMR system in melanoma resistance to TMZ, BCNU, and CDDP, three drugs currently used in the management of this aggressive cancer. To this end, we measured the OGAT and MMR activities in a panel of melanoma cell lines and correlated these values with their sensitivity to the drugs. We also examined the effectiveness of BG in potentiating TMZ or BCNU cytotoxicity.

The nine melanoma cell lines used in the present study displayed a broad range of OGAT activities (Fig. 1A; Table 1). This finding is in agreement with immunohistochemical studies showing considerable variation in OGAT expression in melanoma metastases from different patients (Egyházi et al., 1995). Heterogeneity in OGAT activity was also found at the clonal level (Fig. 2B), confirming with a functional assay the findings of Lee et al. (1992), who detected a marked intercellular variation of enzyme expression in melanoma sections stained with anti-OGAT antibodies.

The melanoma cell lines also showed varying efficiencies of MMR (Fig. 2; Table 1), with two of the nine lines, PR-Mel and PD-Mel, being devoid of MMR activity. PR-Mel was previously shown to be MMR-deficient as a consequence of the loss of hMutLα expression (Alvino et al., 2002), whereas the MMR defect in PD-Mel cells was attributable to the loss of hMHS6 expression. The remaining cell lines fell into two groups. One was fully proficient, with repair levels of about 80%, whereas the other group displayed MMR proficiency of about 40%. In

LCP-Mel cells, the lower MMR efficiency could be associated with the reduced expression of hMSH6, but the reasons underlying the observed MMR attenuation in the CR-Mel and SK-Mel-28 lines require further study, because these cells expressed normal levels of all the tested MMR proteins.

The cytotoxic action of TMZ is primarily due to methylation of guanines at the O^6-position. Because OGAT detoxifies the modified DNA by directly removing the methyl groups from O^6-guanines, high levels of this enzyme confer resistance to this agent. The relationship between OGAT activity and TMZ sensitivity holds also for melanoma cells: as shown in Fig. 3A, we found a direct correlation between TMZ IC_{50} values and OGAT levels. Moreover, LTE to BG potentiated the cytotoxic effects of TMZ in all cell lines, with the exception of those devoid of OGAT activity or deficient for the MMR system (Table 1). The finding that STE to BG failed to increase cellular sensitivity to TMZ can be explained by a rapid enzyme recovery after removal of the inhibitor (Fig. 5). It is noteworthy that all the cell lines analyzed for OGAT activity at clonal level contained a number of clones with enzyme activities significantly higher than those of the corresponding bulk populations (Fig. 1B; Table 1). These clones were also more resistant to TMZ (LCP-Mel clone E3 in Table 1; data not shown). Should a similar situation arise also in melanoma patients, these resistant clones would survive the initial treatment and go on to give rise to a tumor that is refractory to further treatment.

Although OGAT protects cells against the cytotoxicity of methylating agents through detoxifying the DNA, the actual killing process requires a functional MMR system. Unrepaired O^6-MeG residues pair with thymines during replication and the processing of these mispairs by the MMR system triggers the cell-killing process by an as yet uncharacterized mechanism (Karran, 2001). Therefore, resistance to methylating agents can be linked either to high OGAT levels or to loss of MMR. When OGAT activity is attenuated, the number of O^6-MeG/T mispairs increases and the efficiency of cell killing by methylating agents becomes predominantly a function of MMR (Dosch et al., 1998; Marra et al., 2001). This relationship holds also for the majority of the melanoma cell lines analyzed in this study. The fully MMR-proficient lines GL-Mel, M10, and M14 showed the highest sensitivity to TMZ, whereas CR-Mel, LCP-Mel, and its clones E3 and E4, displaying intermediate MMR levels, were about 5-fold less sensitive (Fig. 4A; Table 1). The MMR-deficient lines were highly resistant to TMZ and it is noteworthy that in the absence of MMR, OGAT levels become irrelevant. Thus, PR-Mel and PD-Mel cells have very different OGAT activities, yet both lines were highly resistant to TMZ, and BG failed to increase their sensitivity to the drug (Fig. 4A; Table 1). However, CN-Mel cells were fully MMR-proficient, yet highly resistant to TMZ even after OGAT depletion. In addition, in the presence of BG, SK-Mel-28 cells were about 3-fold more resistant to TMZ than the LCP-Mel and CR-Mel lines, although all three displayed comparable levels of MMR. These data indicate that, in a subset of melanoma cell lines, other factors may affect sensitivity to the drug. TMZ exerts its cytotoxic effects mainly through the induction of apoptosis (Tentori et al., 1995, 1997; D'Atri et al., 1998), and it could be the dysregulation of this that sometimes contributes to TMZ resistance in melanoma.

BCNU treatment gives rise primarily to O^6-chloroethylguanine and OGAT can remove also these bulkier alkyl moieties from guanines to detoxify the DNA. Correspondingly, OGAT has been shown to protect also against chloroethylnitrosourea-induced cytotoxicity (Pegg, 1990; Pegg et al., 1995; Dolan and Pegg, 1997; Gerson, 2002). However, in some tumor cell lines, resistance to BCNU that is not dependent on OGAT has been demonstrated (Silber et al., 1992; Wedge et al., 1996a). Our results (Fig. 3B; Table 1) showed that in melanoma cells OGAT exerts a protective role against BCNU cytotoxicity, because LTE to BG increased sensitivity to the drug in all cell lines with detectable OGAT activity. However, a strong correlation between OGAT levels and IC_{50} values relative to BCNU was not found in this study, suggesting that OGAT is not a major determinant of melanoma resistance to this drug. Besides O^6-chloroethylguanine, BCNU forms other cytotoxic DNA adducts that are removed by base and nucleotide excision repair (Allan et al., 1998), as well as by recombination. The efficiency of these DNA repair pathways, as well as the activity of glutathione *S*-transferase and glutathione levels (Ali-Osman, 1989; Smith et al., 1989), thus also affect the sensitivity of cells to this drug. Our results show that melanoma cells can be protected against killing by BCNU also by the MMR system, albeit only weakly (Fig. 4B). This phenomenon could be linked to the propensity of O^6-(2-chloroethyl)guanine to rearrange to O^6,N^1-ethanoguanine, which in turn reacts with the N^3 of cytosine in the complementary strand to form an interstrand DNA cross-link (Tong et al., 1982). These lesions are generally resolved by recombination processes, which may also involve the MMR system, because MMR was shown to protect some cell lines against killing by CCNU, an analog of BCNU (Aquilina et al., 1998).

The antitumor activity of CDDP depends mainly on the formation of DNA intrastrand and interstrand cross-links (reviewed in Jordan and Carmo-Fonseca, 2000). Cisplatin adducts are mainly repaired by the nucleotide excision repair pathway and enhanced levels of the nucleotide excision repair factors ERCC1 and XPA have been associated with tumor cell resistance to the drug (Jordan and Carmo-Fonseca, 2000). Recently, a deficiency of MMR has also been linked to resistance to CDDP in various ovarian and colon cancer lines (Fink et al., 1998). The analysis of CDDP sensitivity in our panel of melanoma cell lines indicated that MMR status is not a predictor of resistance to CDDP in this system. Thus, although M10, M14, and CN-Mel possessed comparable MMR activities, their sensitivities to CDDP were significantly different. Moreover, the MMR-deficient cell line PD-Mel was one of the more resistant melanoma cell lines, whereas PR-Mel cells displayed an IC_{50} value for CDDP lower than that observed in the MMR-proficient lines. These results seem to reflect the multifactorial nature of CDDP resistance (Akiyama et al., 1999; Jordan and Carmo-Fonseca, 2000).

In conclusion, OGAT and MMR activities are the major, although not exclusive, determinants of sensitivity to TMZ. Pretherapy determination of melanoma OGAT expression and MMR status might therefore be useful to identify patients most likely to benefit from TMZ treatment. Furthermore, a BG treatment schedule that ensures a prolonged depletion of OGAT might significantly increase TMZ efficacy in MMR-proficient melanomas. In the case of BCNU, the situation is not so clear-cut. Thus, although OGAT does protect against BCNU cytotoxicity, resistance to this drug can be

linked to other pathways of DNA metabolism and DNA damage signaling, such that OGAT activity of tumor cells is not a reliable marker of patient responsiveness to BCNU. Moreover, the use of BCNU in combination with BG might be of clinical relevance only in those patients whose melanoma is resistant to BCNU as a consequence of its high OGAT activity. Unlike in other cell types, MMR efficiency does not seem to affect the sensitivity of melanoma cells to CDDP, and the determination of MMR status in these tumors therefore seems to be of no clinical relevance.

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