Combined effect of temozolomide and hyperthermia on human melanoma cell growth and O[6]-methylguanine-DNA methyltransferase activity

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Abstract. Hyperthermic isolated limb perfusion (HILP) with L-phenylalanine mustard (L-PAM) represents an effective treatment for locally advanced melanoma of the limbs. However, regional chemotherapy of melanoma still needs to be improved. Temozolomide (TMZ) is a methylating agent that spontaneously decomposes into the active metabolite of dacarbazine, the most effective agent for the systemic treatment of melanoma. Tumor cells with high levels of O[6]-methylguanine-DNA methyltransferase (MGMT) and/or with a defective DNA mismatch repair (MMR) are resistant to TMZ. Inhibition of MGMT activity increases TMZ sensitivity of MMR-proficient, but not of MMR-deficient cells, while inhibition of base excision repair (BER) potentiates TMZ cytotoxicity in both cell types. Recent studies, performed in an animal model, have shown that TMZ is more effective than L-PAM when applied regionally and that hyperthermia can increase the antitumor activity of TMZ. In this study, three thermoresistant human melanoma cell lines, endowed with different MGMT activity and functional status of the MMR system, were treated with TMZ at 37°C or 41.5°C for 90 min, and then analyzed for cell growth and MGMT activity. Hyperthermia significantly enhanced TMZ cytotoxicity in MMR-proficient cells, either endowed or not with MGMT activity, and in MMR-deficient cells. Endogenous MGMT activity was not affected by hyperthermia that, however, enhanced the enzyme depletion induced by TMZ treatment. Moreover, MGMT recovery after drug removal was delayed in cells that had been treated at 41.5°C. Taken together, these findings confirm the therapeutic potential of a combined treatment of hyperthermia and TMZ. They also suggest that inhibition of BER and/or increased DNA methylation may be involved in the thermal enhancement of TMZ cytotoxicity. Additional studies are necessary to better clarify the mechanisms underlying hyperthermia-induced potentiation of TMZ activity. However, the present investigation provides further support to the development of clinical trials of HILP with TMZ.

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Introduction

Isolated limb perfusion (ILP) with cytotoxic drugs represents an effective form of treatment for patients with regionally advanced melanoma confined to an extremity (reviewed in refs. 1-3). This procedure has the benefit of delivering high dose of chemotherapeutic agents with acceptable local toxicity and minimal systemic side effects (1-3). In recent years, a less invasive modality of ILP, named isolated limb infusion (ILI) has been developed, which appears to possess the same efficacy as ILP (2,4). Among the antitumor drugs that have been studied in ILP and ILI, melphalan (L-phenylalanine mustard, L-PAM) has emerged as the most effective agent and presently it is the most used drug in the regional treatment of melanoma of the limbs (1-3). The overall response rate for ILP with L-PAM is in the 70-80% range, with 30-50% of complete responses, and a median duration of local tumor control of six months (1-3).

Hyperthermia is a therapeutic procedure used to raise the temperature of a region of the body affected by cancer, and it is usually administered along with other treatment modalities (reviewed in refs. 5-8). Temperatures in the range of 39-40°C are considered 'mild hyperthermia', while temperatures in the range of 41-43°C are considered 'true hyperthermia' (8). The introduction of hyperthermia in the clinical setting was based on preclinical studies showing that temperatures above 41-42°C can induce direct tumor cell-killing, and that even mild hyperthermia can increase the therapeutic effects of radiation and cytotoxic agents (6,7).

Hyperthermia is frequently used in conjunction with ILP to improve the response to regional chemotherapy of melanoma (reviewed in refs. 1-9). Presently, it is generally accepted that tissue temperature should not exceed 42˚C during HILP. Indeed, although administration of L-PAM at tissue temperatures above 42˚C produces the best tumor responses, it is associated with a high incidence of severe local toxicity (9,10). Clinical studies in which hyperthermia in the range of 39-42˚C has been added to ILP with L-PAM have shown an overall response rate between 80 and 100% and complete response rates between 40 and 82% (2,9,11). The median response duration is typically 9-12 months, with a subgroup of complete responders showing sustained complete responses (2,8,11).

The molecular mechanisms underlying the ability of hyperthermia to increase cell sensitivity to cytotoxic agents and radiation are not fully elucidated yet. Enhanced drug uptake (12), increased formation of DNA interstrand cross-links and inhibition of their repair (13), impairment of cellular detoxification systems (14), inhibition of the $p34^{cdc2}$ kinase (15) have been implicated in the thermal enhancement of L-PAM activity. Inhibition of DNA repair appears also involved in the thermal enhancement of cisplatin cytotoxicity (16) and in hyperthermia-induced radiosensitization (17,18).

Temozolomide (TMZ) is a methylating agent that spontaneously decomposes into 5-(3-methyl-1-triazeno)imidazole-4-carboxamide, the active metabolite of dacarbazine (19). 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 (20). TMZ has activity comparable to that of dacarbazine in melanoma (20,21). However, unlike dacarbazine, TMZ penetrates the blood-brain barrier, which can be beneficial in preventing or treating melanoma metastases to the central nervous system (20,21).

The most frequent sites of methylation by TMZ are the N^7 position of guanine (N^7-G, 70% of total adducts), the N^3 position of adenine (N^3-A, 10% of total adducts) and the O^6 position of guanine (O^6-G, 5% of total adducts) (22). Methyl adducts at N^7-G and N^3-A are efficiently removed by the base excision repair (BER) pathway (reviewed in ref. 23) and contribute little to TMZ cytotoxicity, unless cells are exposed to high drug concentrations (24) or to inhibitors of BER (25). Cytotoxicity of TMZ concentrations that can be achieved after systemic administration of the drug is primarily due to the methylation of O^6-G. 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-methyl-guanine-DNA methyltransferase (MGMT, E.C.2.1.1.63). MGMT 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. 26-28). Consistently, depletion of MGMT activity by the competitive inhibitor O^6-benzylguanine (reviewed in ref. 29) increases tumor cell sensitivity to TMZ both *in vitro* and *in vivo* (22,26-29).

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 refs. 30,31). According to the 'futile repair' model (32,33) 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. 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 (24,34-37). This is followed by either apoptosis (24,34,37-39), mitotic catastrophe, or a senescence-like state (35,36). According to the 'signaling' model (40) 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 O^6-benzylguanine fails to increase drug sensitivity in these cells (22,41). Notably, inhibition of BER increases sensitivity to methylating agents in both MMR-proficient and MMR-deficient tumor cells (25).

Recent studies performed by Ueno *et al* (42) in a rodent animal model of in-transit melanoma have shown that TMZ is more effective than L-PAM when used in ILI regional treatment. Moreover, in the same animal model, Ko *et al* (43) have demonstrated that strong synergistic antitumor effects occur when ILI with TMZ is performed under hyperthermic conditions.

The present investigation was performed to further evaluate the therapeutic potential of a combined treatment of hyperthermia and TMZ, and to get insight into the possible mechanisms underlying the thermal enhancement of the drug cytotoxicity. To this end, we evaluated the effects of hyperthermia on TMZ sensitivity of melanoma cell lines endowed with different MGMT levels and functional status of the MMR system. Moreover, we investigated whether MGMT basal activity, and/or depletion after TMZ treatment and/or recovery after drug removal could be affected in cells exposed to hyperthermia.

Materials and methods

Cell lines and culture conditions. Three thermoresistant human melanoma cell lines were used in this study. M14 (44) was kindly provided by Gabriella Zupi (Istituto Regina Elena, Rome, Italy); PR-Mel (45,46) was established in our laboratory; GL-Mel-D_2, a clonal melanoma cell line, was derived by limiting dilution from the GL-Mel cell line (46), kindly provided by Fiorella Guadagni (Regina Elena Cancer Institute, Rome, Italy). M14 and PR-Mel were established from cutaneous metastases, while GL-Mel was derived from a lymph node metastasis. GL-Mel and M14 are MMR-proficient (46), while PR-Mel is MMR-deficient (45,46). Expression of MMR repair protein in M14, PR-Mel and GL-Mel-D_2 is illustrated in Fig. 1. MGMT activity of the three melanoma cell lines is reported in Table I.
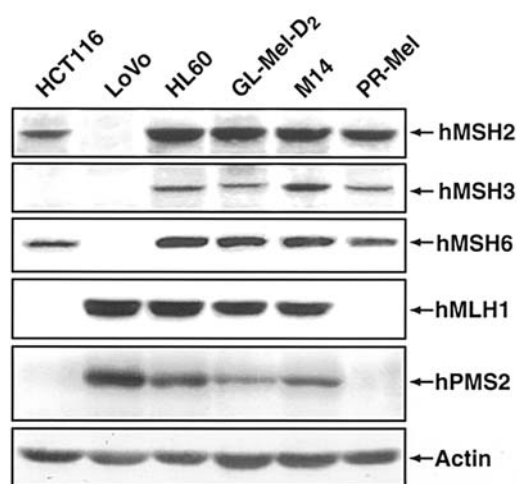
Figure 1. MMR protein expression in human melanoma cell lines. Whole cell extracts (60 µg) were subjected to electrophoresis on a 7% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with antibodies against hMSH2, hMSH3, hMSH6, hMLH1 or hPSM2. Incubation with anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. Controls, LOVO cells, expressing hMLH1 and hPSM2 but not hMSH2 and hMSH6; HCT116 cells, expressing hMSH2 and hMSH6 but not hMLH1 and hPMS2; MMR-proficient cell line HL60.

The cell lines were 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 anti-biotics (Invitrogen, Paisley, Scotland), hereafter referred to as complete medium (CM).

Drugs and reagents. TMZ was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). L-PAM, and 3-(4,5-dimethyl-thiazol-2-yl))-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

TMZ was prepared freshly in CM (388 µg/ml) and discarded after use. L-PAM solution was also prepared freshly

and discarded after use. The drug was dissolved in ethanol (380.8 µg/ml), and then diluted in CM. The final concentrations of ethanol in the cultures treated with L-PAM did not affect cell growth (data not shown). MTT was prepared 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; mAb against actin (clone AC-40) came from Sigma-Aldrich; 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.

Western blot analysis. Cells were suspended in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM AEBSF, and 1X protease inhibitor cocktail Complete EDTA-free (Roche Diagnostic, GmbH, Mannheim, Germany)] for 10 min on ice. Cell lysates were then clarified by centrifugation, diluted in 5X Laemmli sample buffer and boiled for 5 min. Protein, 60 µg per sample, was run on 7% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences), and blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween-20, and 5% 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),

Table I. Effect of hyperthermia on melanoma cell sensitivity to TMZ and L-PAM.

Cell line[a]	MGMT[b]	MMR[c]	TMZ IC$_{50}$ (µM)[d]			L-PAM IC$_{50}$ (µM)[d]		
			37˚C	41.5˚C	P-value[e]	37˚C	41.5˚C	P-value[e]
M14	136±14	+	398±33	157±9	<0.01	12.0±0.5	2.2±0.2	<0.01
GL-Mel-D$_2$	ND[f]	+	310±9[g]	247±21	<0.05	29.7±2.3	12.2±1.4	<0.01
PR-Mel	15±3	-	>1000	483±44	<0.01	29.8±5.0	13.4±0.4	<0.01

[a]Cells were exposed to graded concentrations of TMZ or L-PAM at 37˚C or 41.5˚C for 90 min, washed and then plated in flat-bottom 96-well plates as described in Materials and methods. After 5 days of culture at 37˚C, cell proliferation was evaluated by the MTT assay. [b]MGMT activity is expressed in terms of fmoles of ^{3}H-methyl groups transferred per mg of protein in cell extract. Each value represents the arithmetic mean ± standard error of the mean of at least three independent experiments. [c]+, MMR-proficient cells; -, MMR-deficient cells. [d]Drug concentration required to inhibit cell growth by 50%. Each value represents the arithmetic mean ± standard error of the mean of three independent experiments. [e]Probability calculated according to Student's t-test comparing the IC$_{50}$ values obtained at 37˚C with those obtained at 41.5˚C. [f]ND, not detectable. [g]P<0.05 according to Student's t-test comparing the IC$_{50}$ value of GL-Mel-D$_2$ with that of M14.

anti-hMSH3 (1:6000), anti-hMSH6 (0.5 μg/ml), anti-hMLH1 (2 μg/ml), anti-PMS2 (0.5 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.

Drug treatment under normothermic or hyperthermic conditions and cell growth evaluation by the MTT assay. Melanoma cells were removed from continuous culture, suspended (1x10^6 cells/ml) in CM and exposed to graded concentrations of TMZ (125-1000 μM) or L-PAM (1.95-62.5 μM) at 37˚C or 41.5˚C for 90 min into a gently shaken and temperature-controlled water bath. At the end of incubation, cells were washed and plated (2x10^3 cells/well, in a final volume of 0.1 ml of CM) in flat-bottom 96-well plates (Falcon, BD Biosciences Discovery Labware, Bedford, MA). Plates were then incubated at 37˚C for 5 days. Six replicate wells were used for controls and each drug concentration. Control groups for L-PAM were treated with ethanol alone.

The MTT assay was performed as previously described (47). 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, either at 37˚C or 41.5˚C, was expressed in terms of IC_{50} (i.e. 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). Differences between IC_{50} values obtained at 37˚C and IC_{50} values obtained at 41.5˚C were subjected to statistical analysis according to Student's t-test.

Colony formation assay. M14 cells were exposed to graded concentrations of TMZ (125-1000 μM) at 37˚C or 41.5˚C for 90 min, as described for the MTT assay. The cells were then washed twice with PBS, seeded in triplicate into 100x20 mm dishes (Falcon) (100-400 cells/dish) and cultured at 37˚C for 12-15 days. At the end of the assay, colonies were fixed and stained with crystal violet for visualization. Only colonies comprising ≥50 cells were counted. Colony formation efficiency was calculated as the ratio between the number of colonies and the number of cells plated. TMZ IC_{50} values were calculated on the regression line in which the values of colony formation efficiencies were plotted against the logarithm of drug concentration. Differences between IC_{50} values obtained at 37˚C and IC_{50} values obtained at 41.5˚C were subjected to statistical analysis according to Student's t-test.

MGMT assay. MGMT activity in total cell extracts was determined by measuring the transfer of ^3H-methyl groups from a DNA substrate to the MGMT protein as previously described (46), and expressed in terms of fmoles of ^3H-methyl groups transferred per mg of protein in cell extract.

To investigate the effect of hyperthermia on MGMT basal activity, depletion after TMZ treatment and recovery during culture in drug-free medium, M14 cells were incubated in CM or CM containing TMZ (125-1000 μM) at 37˚C or 41.5˚C for 90 min, washed twice with PBS, and cultured at 37˚C for 48 h. MGMT activity was assayed at the end of treatment and after 24 h and 48 h of culture. MGMT activity in TMZ-treated samples was expressed as percentage of the enzyme activity detected, at the same time-point, in the corresponding groups not exposed to the drug. Statistical analysis was conducted on percentages of MGMT activity with respect to appropriate controls. Therefore, in order to process normally distributed data, experimental values were subjected to angular transformation. Thereafter, classical Student's t-test analysis was performed on transformed data.

Results

Combined effect of TMZ and hyperthermia on melanoma cell proliferation and survival. To examine the effects of a combined treatment of TMZ and hyperthermia on human melanoma cell growth, we used three thermoresistant melanoma cell lines endowed with different MGMT activity and functional status of the MMR system.

The cells were treated with graded concentrations of TMZ either at 37˚C or 41.5˚C for 90 min, washed, and then assayed for proliferation after 5 days of culture at 37˚C. Since it is well established that hyperthermia enhances cytotoxicity of L-PAM (2,9-11), as a control, melanoma cells were exposed to graded concentrations of the drug under the same experimental conditions.

The results obtained under normothermic conditions confirm previous findings showing that MGMT activity and a defective MMR system confer protection against TMZ cytotoxicity. Indeed, the MMR-proficient cell line M14, endowed with moderate levels of MGMT activity, was less sensitive to TMZ treatment than the MMR-proficient cell line GL-Mel-D_2, devoid of detectable enzyme activity (Table I). On the other hand, MMR-deficient PR-Mel cells, although possessing extremely low levels of MGMT activity, were highly resistant to TMZ (Table I).

Exposure of melanoma cells to TMZ under hyperthermic conditions resulted in a higher cell growth inhibition with respect to that obtained at 37˚C (Table I). TMZ sensitivity of both M14 and PR-Mel cell lines increased more than 2-fold when drug exposure occurred at 41.5˚C, whereas sensitivity of GL-Mel-D_2 cell line was less affected, with an increase of about 20%.

Consistent with previous findings, we observed that hyperthermia was able to enhance the inhibitory effects of L-PAM on melanoma cell growth (Table I). M14 cells were the most sensitive to the drug and under hyperthermic conditions showed a 5-fold reduction of the IC_{50} value. GL-Mel-D_2 and PR-Mel cell lines displayed comparable sensitivity to L-PAM and drug cytotoxicity was increased about 2-fold in both cell lines.

Fig. 2 shows, for the three melanoma cell lines, the concentration-response curves obtained for TMZ and L-PAM, either at 37˚C or 41.5 ˚C, in one of the independent experiments used to calculate the mean drug IC_{50} values reported in Table I.

To further confirm the ability of hyperthermia to increase melanoma cell sensitivity to TMZ, we evaluated clonogenic
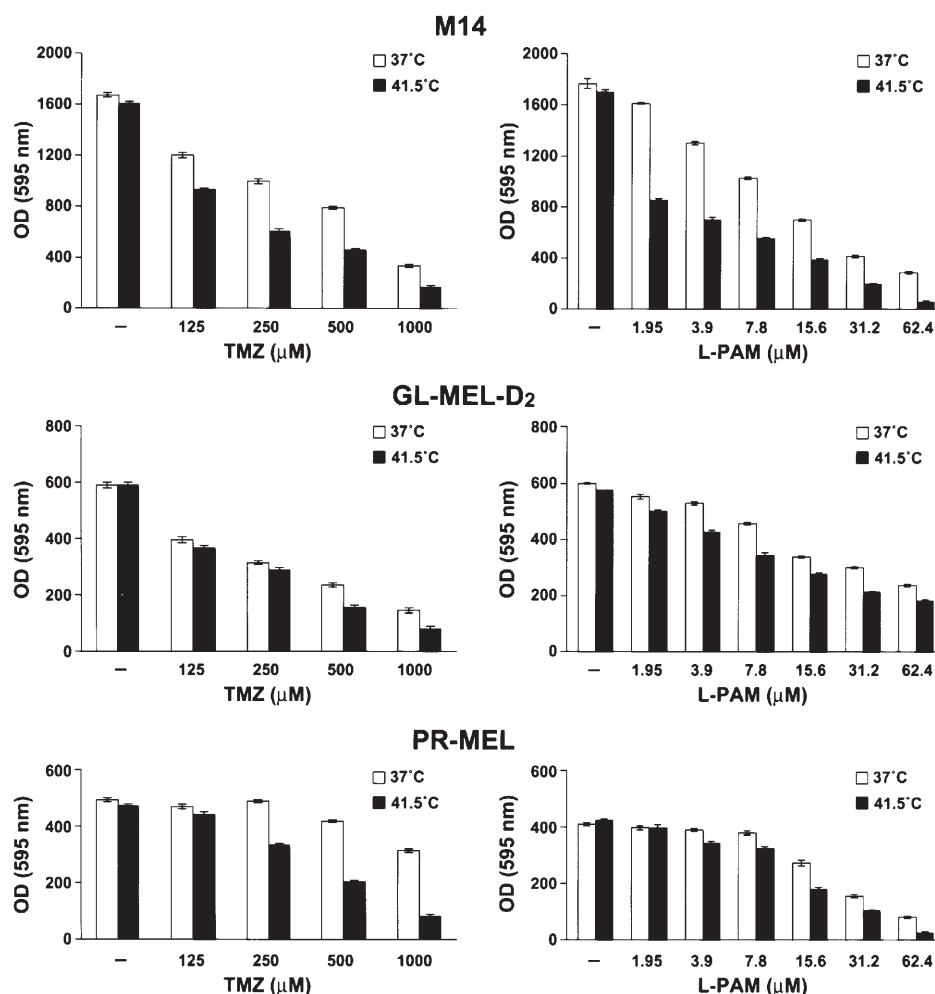
Figure 2. Concentration-response diagrams relative to melanoma cell lines exposed to TMZ or L-PAM under normothermic or hyperthermic conditions. Melanoma cells were exposed to the indicated concentrations of TMZ or L-PAM at 37˚C or 41.5˚C for 90 min. At the end of incubation, the cells were washed and plated in flat-bottom 96-well plates as described in Materials and methods. Cell growth was evaluated by the MTT assay after 5 days of culture at 37˚C. Data are expressed in terms of adsorbance at 595 nm. Each value represents the arithmetic mean ± standard error (SE) of the mean of six replicate wells. Data refer to a representative experiment.

survival of M14 cells exposed to the drug at 37˚C or 41.5˚C. As shown in Fig. 3, hyperthermia alone did not affect survival of M14 cell line, whereas TMZ alone induced a concentration-dependent inhibition of M14 clonal growth. When M14 cells were treated with TMZ under hyperthermic conditions, a significant increase in TMZ-induced suppression of colony formation was observed at all the concentrations tested.

Combined effect of TMZ and hyperthermia on MGMT activity of melanoma cells. Experiments were performed to investigate whether MGMT basal activity, and/or depletion after TMZ treatment and/or recovery after drug removal could be affected in melanoma cells exposed to hyperthermia. Cells of M14 line were incubated in CM or CM containing graded amounts of TMZ at 37˚C or 41.5˚C for 90 min, washed and then assayed for MGMT activity at the end of treatment and after 24 h and 48 h of culture at 37˚C.

Hyperthermia alone did not affect MGMT activity, either at the end of the hyperthermic treatment or at 24 h or 48 h of further normothermic culture (see legend of Fig. 4).

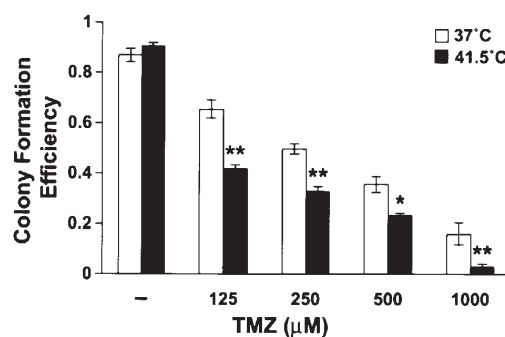When M14 cells were treated with TMZ at 37˚C and assayed for MGMT activity at the end of drug exposure, the

Figure 3. Colony formation efficiency of M14 cell line exposed to TMZ alone or combined with hyperthermia. Cells were suspended in CM, exposed to the indicated concentrations of TMZ at 37˚C or 41.5˚C for 90 min, washed, and seeded into 100x20 mm dishes as described in Materials and methods. Colony formation efficiency was evaluated after 12-15 days of culture at 37˚C and 41.5˚C. Each value represents the arithmetic mean ± SE of the mean of three independent experiments performed with triplicate samples. $^{*}P<0.05$; and $^{**}P<0.01$, according to Student's t-test comparing the colony formation efficiency of M14 cell line treated with TMZ at 41.5˚C with that of the cell line treated with the drug at 37˚C. The means of TMZ IC_{50} values relative to the three independent experiments were 330±25.9 at 37˚C and 121.3±2.7 at 41.5˚C ($P<0.01$, according to Student's t-test analysis).
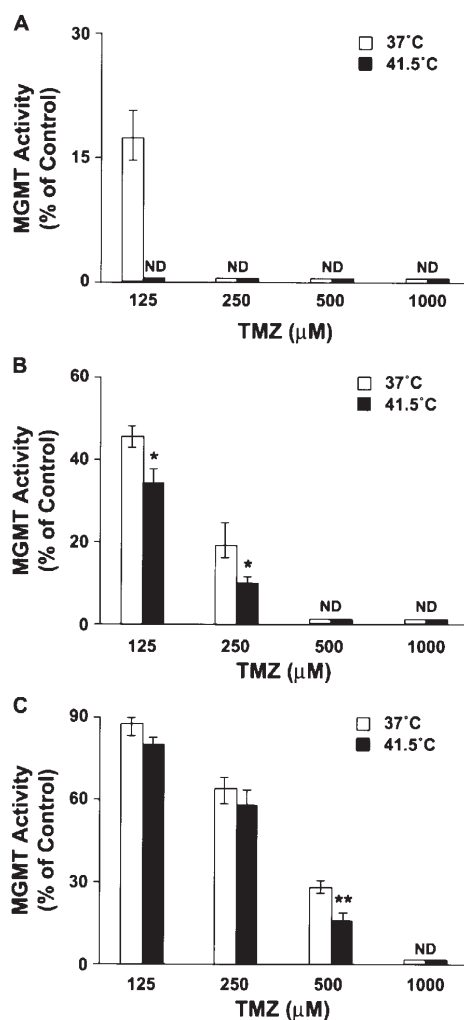
Figure 4. TMZ-induced depletion of MGMT activity and kinetics of enzyme recovery in M14 cells exposed to the drug under normothermic or hyperthermic conditions. Melanoma cells were suspended in CM or CM containing the indicated concentrations of TMZ, and incubated at 37°C or 41.5°C for 90 min. Thereafter, the cells were washed, and cultured at 37°C for 48 h. MGMT activity was assayed at the end of treatment (A), and after 24 h (B) and 48 h (C) of normothermic culture. For each time-point, MGMT activity of TMZ-treated groups was expressed as percentage of enzyme activity detected in the cells incubated in CM alone at the same temperature. Values represent the means obtained after angular transformation of the data (see Materials and methods), and the upper and lower limit of the mean relative to three independent experiments. *P<0.05; and **P<0.01, comparing the percentage of MGMT activity of M14 cells treated with TMZ at 41.5°C with that of the cells treated with the drug at 37°C. Arithmetic mean (± SE) of MGMT activity in M14 cells incubated in CM alone at 37°C for 90 min was 150±20 fmoles/mg protein at the end of treatment, 157±33 fmoles/mg protein after 24 h of culture and 141±26 fmoles/mg protein after 48 h of culture. MGMT activity in M14 cells incubated in CM alone at 41.5°C for 90 min was 148±21 fmoles/mg protein at the end of treatment, 143±31 fmoles/mg protein after 24 h of culture and 136±27 fmoles/mg protein after 48 h of culture. These values of MGMT activity are not statistically different, according to Student's t-test analysis.

enzyme activity was markedly reduced at the concentration of 125 μM and no longer detectable at higher concentrations (Fig. 4A). These results are in agreement with previous findings showing that in cells exposed to TMZ, a depletion of MGMT activity occurs as a consequence of enzyme inactivation during repair of methyl adducts at O^6-G (48-50). A TMZ-induced depletion of MGMT activity was also observed in M14 cells

exposed to the drug under hyperthermic conditions. In this case, however, no enzyme activity was detectable at the concentration of 125 μM (Fig. 4A).

After 24 h of culture in drug-free medium, M14 cells exposed to 125 and 250 μM TMZ under normothermic conditions showed a partial and concentration-dependent recovery of MGMT activity, while the enzyme activity remained undetectable in cells treated with TMZ 500 μM and 1000 μM (Fig. 4B). M14 cells treated with the drug at 41.5°C also displayed a partial recovery of MGMT activity at the concentrations of 125 and 250 μM. However, the enzyme recovery was significantly, although moderately, lower than that observed in the cells exposed to the drug under normothermic conditions (Fig. 4B).

A further recovery of MGMT activity was detected in TMZ-treated cells after 48 h of culture (Fig. 4C). In this case, cells exposed to 125 and 250 μM TMZ under normothermic or hyperthermic conditions did not show differences in MGMT activity. However, MGMT recovery in M14 cells treated with 500 μM TMZ at 41.5 °C was lower with respect to that of the cells treated with the same drug concentration at 37°C. No MGMT activity was detectable in cells treated with 1000 μM TMZ, either at 37°C or at 41.5 °C (Fig. 4C).

Discussion

HILP with L-PAM is presently considered the best therapy for local advanced melanoma of the limbs. However, the consistent number of partial responders, the lack of response durability in a large proportion of patients, and the moderate limb toxicity associated with this treatment modality highlight the necessity to develop more effective, durable and less toxic regional therapeutic strategies.

The methylating agent TMZ appears to be a good candidate for use in conjunction with hyperthermia for regional chemotherapy of melanoma. Indeed, recent studies performed in a rat model of in-transit melanoma have shown that TMZ is more effective than L-PAM when used in ILI, and that hyperthermia can significantly increase the antitumor activity of TMZ in a regional setting (42,43).

In this study, performed on three different thermoresistant human melanoma cell lines, we further confirm that under hypertermic conditions adopted in several clinical protocols of HILP (2,8-10) the cytotoxic effects of TMZ are significantly increased. Moreover, we show that thermal enhancement of TMZ cytotoxicity occurs in MMR-proficient cells, either endowed or not with appreciable MGMT activity (i.e M14 and GL-Mel-D_2 cells, respectively), and in MMR-deficient cells (i.e PR-Mel cells). We also demonstrate that MGMT basal activity is not affected by the hyperthermic conditions used in the study. However, the enzyme depletion induced by TMZ appears to be enhanced by hyperthermia, whereas MGMT recovery after drug removal is delayed in cells which have been treated with the drug under hyperthermic conditions. Taken together, our findings are compatible with the hypothesis that inhibition of BER and/or increased total DNA methylation underlie, at least in part, the thermal enhancement of TMZ cytotoxicity.

The BER pathway is responsible for the removal of a wide spectrum of small DNA lesions, including those induced

by alkylating agents, reactive oxygen species, and other reactive metabolites capable of modifying DNA bases (23). In the short-patch BER, the damaged base is removed by a lesion-specific glycosylase. Subsequently, the resulting abasic site is recognized by apurinic/apyrimidinic endonuclease, which incises the damaged strand, producing a strand break with a 5' terminal deoxyribose-phosphate moiety. Thereafter, DNA polymerase ß (pol ß) catalyzes release of the phospho-deoxyribose moiety filling the single nucleotide gaps; the nick is then sealed by DNA ligase I or III. A second pathway of BER, called long-patch BER, involves DNA synthesis to fill a gap of 2-13 nucleotides. Substantial experimental evidence indicates that pol ß plays a crucial role also in this long-patch pathway (51). The enzyme poly(ADP-ribose) polymerase-1 (PARP-1) (reviewed in ref. 52) participates in both short- and long-patch BER after nick formation. Upon binding to DNA strand breaks, PARP-1 becomes activated and synthesizes homopolymers of ADP-ribose from NAD+ to modify itself and other nuclear acceptor proteins (e.g. histones) (52,53). The negatively charged polymers cause the electro-static repulsion of histones from DNA and favour the recruitment of the other BER components to complete the repair process (52,53).

A large body of experimental evidence has demonstrated that cytotoxicity of methylating agents increases when the BER pathway is impaired. Mouse fibroblasts deficient in pol ß are hypersensitive to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate (54); HeLa cells expressing apurinic/apyrimidinic endonuclease antisense RNA show increased sensitivity to methyl methanesulfonate (55); PARP-/- mice, and cells derived from them, display high sensitivity to *N*-methyl-*N*-nitrosourea (56). Moreover, pharmacological disruption of BER by PARP-1 inhibitors or methoxyamine, greatly potentiates the cytotoxic effects of various methylating agents, including TMZ, in both MMR-proficient and MMR-deficient tumor cells (25). Interestingly, previous studies have shown that the activity of pol ß is markedly impaired in cells exposed to 42-45˚C (57,58). Moreover, a reduction in the levels of NAD+, leading to a decrease of PARP-1 activity of ~50%, has been demonstrated in cells heated at 41.8˚C (59). It is therefore possible to hypothesize that hyperthermia may potentiate TMZ cytotoxicity through an indirect inhibition of PARP-1 function and/or through a direct impairment of pol ß activity.

Increased DNA alkylation has been implicated in the thermal enhancement of L-PAM and BCNU activity (60-62). It is possible that the number of methyl adducts formed in cells exposed to TMZ also increases when the drug is given in conjunction with hyperthermia. This could result in the potentiation of TMZ cytotoxicity in both MMR-proficient and MMR-deficient cells. Indirect support to this hypothesis comes from the analysis of MGMT depletion and recovery in the M14 cell line treated with the drug under normothermic or hyperthermic conditions. In agreement with previous studies (63), we found that MGMT activity was not influenced by hyperthermia alone. However, while in cells exposed to 125 μM TMZ under normothermic conditions residual MGMT activity (~15% of untreated control) was still detectable at the end of treatment, no MGMT activity could be evidenced in the cells treated with this drug concentration at 41.5˚C.

Moreover, in cells exposed to 125 and 250 μM TMZ at 41.5˚C and cultured in drug-free medium for 24 h, as well as in cells treated with 500 μM TMZ at the same temperature and cultured for 48 h, MGMT activity was significantly lower than that observed in the cells subjected to the drug under normothermic conditions. MGMT-mediated repair of methyl adducts at O^6-G occurs through a stoichiometric and auto-inactivating reaction, and *de novo* enzyme synthesis is required to restore cellular MGMT levels (26-28). Therefore, in cells exposed to a methylating agent, the reduction of MGMT activity at the end of treatment is dependent on the amount of methyl adducts formed at O^6-G. Moreover, in cells in which the initial MGMT levels are not sufficient to repair all methyl adducts formed at O^6-G, a delayed recovery of enzyme activity after removal of the drug is expected, since the *de novo* synthetized protein undergoes inactivation upon repair of residual methyl adducts. Our findings support therefore the hypothesis that the amount of methyl adducts formed at O^6-G, and reasonably at different positions of DNA bases, is higher in cells exposed to TMZ under hyperthermic conditions than in cells treated with the drug at 37˚C.

A number of other mechanisms could be envisaged to explain the effect of hyperthermia in the present experimental model. For example, signal transduction pathways that regulate cell cycle arrest, DNA repair, apoptosis and survival could be involved. However, the data presently available on possible interaction between temozolomide and hyperthermic treatment, do not allow to advance further hypotheses based on direct or indirect experimental evidence.

In conclusion, this study confirms that TMZ cytotoxicity is significantly increased by hyperthermia and shows that the thermal enhancement of drug activity occurs not only in MMR-proficient cells, but also in MMR-deficient cells, which are highly resistant to TMZ. It also suggests that inhibition of BER and/or increased DNA methylation may be involved in the thermal enhancement of TMZ cytotoxicity. Although further investigations are necessary to better define the molecular mechanisms underlying the thermal enhancement of TMZ cytotoxicity, our findings provide further support to the development of clinical trials of HILP with TMZ.

Acknowledgements

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