Temozolomide Pharmacodynamics in Patients with Metastatic Melanoma: DNA Damage and Activity of Repair Enzymes O^6-Alkylguanine Alkyltransferase and Poly(ADP-Ribose) Polymerase-1

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Abstract

Purpose: Temozolomide, a DNA methylating agent used to treat melanoma, induces DNA damage, which is repaired by O^6-alkylguanine alkyltransferase (ATase) and poly(ADP-ribose) polymerase-1 (PARP-1)–dependent base excision repair. The current study was done to define the effect of temozolomide on DNA integrity and relevant repair enzymes as a prelude to a phase I trial of the combination of temozolomide with a PARP inhibitor.

Experimental Design: Temozolomide (200 mg/m^2 oral administration) was given to 12 patients with metastatic malignant melanoma. Peripheral blood lymphocytes (PBL) were analyzed for PARP activity, DNA single-strand breakage, ATase levels, and DNA methylation. PARP activity was also measured in tumor biopsies from 9 of 12 patients and in PBLs from healthy volunteers.

Results: Temozolomide pharmacokinetics were consistent with previous reports. Temozolomide therapy caused a substantial and sustained elevation of N^7-methylguanine levels, a modest and sustained reduction in ATase activity, and a modest and transient increase in DNA strand breaks and PARP activity in PBLs. PARP-1 activity in tumor homogenates was variable (828 ± 599 pmol PAR monomer/mg protein) and was not consistently affected by temozolomide treatment.

Conclusions: The effect of temozolomide reported here are consistent with those documented in previous studies with temozolomide and similar drug, dacarbazine, demonstrating that a representative patient population was investigated. Furthermore, PARP activity was not inhibited by temozolomide treatment and this newly validated pharmacodynamic assay is therefore suitable for use in a proof-of-principle phase I trial a PARP-1 inhibitor in combination with temozolomide.

Intrinsic or acquired resistance remains a major limitation in the use of cytotoxic chemotherapy. A variety of cancer cell resistance mechanisms have been described or proposed, including decreased drug uptake into cells, increased drug efflux intracellular drug inactivation, alteration of the cellular target, repair of drug-induced damage, or development of tolerance to drug-induced lesions (1). Strategies directed at overcoming these mechanisms of drug resistance are being evaluated, including the targeting of DNA repair pathways, in an attempt to improve on the efficacy of existing cytotoxic drugs.

Temozolomide is an orally available monofunctional DNA alkylating agent used to treat gliomas and malignant melanoma (2). Temozolomide is rapidly absorbed and undergoes spontaneous breakdown to form the active monomethyl triazene, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide. Monomethyl triazene forms several DNA methylation products, the predominate species being N^7-methylguanine (70%), N^3-methyladenine (9%), and O^6-methylguanine (5%; ref. 3). Unless repaired by O^6-alkylguanine alkyltransferase (ATase; ref. 3), O^6-methylguanine is a cytotoxic lesion due to mispairing with thymine during DNA replication (4). This mispairing is recognized on the daughter strand by mismatch repair proteins and the thymine excised. However, unless the original O^6-methylguanine lesion is repaired by ATase-mediated removal of the methyl adduct, thymine can be reinserted. Repetitive futile rounds of thymine excision and incorporation opposite an unrepaired O^6-methylguanine lesion causes a state of persistent strand breakage and the MutS branch of mismatch repair

system signals G_2-M cell cycle arrest and the initiation of apoptosis (5–7). The quantitatively more important N^7-methylguanine and N^3-methyladenine lesions formed by temozolomide are rapidly repaired by base excision repair.

The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) plays a key role in facilitating base excision repair (8) and other cellular processes (reviewed in ref. 9). It has been proposed that PARP-1 acts as a molecular DNA nick sensor, detecting DNA single-strand breaks and recruiting the appropriate repair enzymes (10). PARP-1 binds to DNA strand breaks via two zinc fingers in the amino-terminal DNA binding domain of the enzyme, its activity being dependent on DNA binding (11). The enzyme acts as a homodimer (12) catalyzing the transfer of ADP-ribose from the substrate NAD^+ to acceptor proteins, including PARP-1 itself. Extensive negatively charged polymers of PAR are thereby formed, causing electrostatic repulsion of DNA strands and chromatin proteins, the latter allowing base excision repair complexes access to the damaged strand and subsequent DNA repair. After initial activation by a strand break, PARP-1 is released from the DNA, the polymer degraded by PAR glycohydrolase, and the PARP-1 enzyme is then available for a further round of DNA binding and activation (13). There is considerable interest in the development of PARP inhibitors as both chemopotentiators and radiopotentiators for use in cancer therapy and to limit cellular damage after ischemia or endotoxic stress (14). In particular, potentiation of temozolomide cytotoxicity observed in preclinical studies with potent PARP-1 inhibitors reflects inhibition of base excision repair and subsequent cytotoxicity due to incomplete processing of N^7-methylguanine and N^3-methyladenine.

There is now a large body of preclinical data demonstrating that the cytotoxicity of temozolomide is potentiated by co-administration of a PARP inhibitor either *in vitro* (15–17) or *in vivo* (18, 19). A phase I trial of a novel PARP inhibitor in combination with temozolomide began in 2003 at three Cancer Research UK clinical centers in collaboration with Pfizer GRD (La Jolla, CA). Demonstration of PARP inhibition and potentiation of temozolomide-induced DNA damage are key elements of this phase I trial for which a validated PARP activity assay for use on clinical material was required.

The aims of our study were to validate our assay for PARP activity on clinical material and to describe the pharmacodynamic effects of temozolomide in melanoma patients. Peripheral blood lymphocytes (PBL) were obtained for the analysis of PARP activity, DNA single-strand breaks, N^7-methylguanine levels, and ATase activity before and after temozolomide administration. PARP activity was also assessed in PBLs from healthy volunteers and in tumor biopsy specimens taken before and after temozolomide. These data will allow detailed mechanistic assessment of putative DNA repair inhibitors used in conjunction with temozolomide from trials now under way.

Materials and Methods

Validated and published methods were used for the measurement of temozolomide plasma concentrations (20), ATase activity (21), DNA single-strand breaks by the alkaline COMET assay (22), and DNA methylation. COMET assay results are expressed in terms of the Olive moment, which is defined as tail length × %DNA in tail/100, and gives a measure of the amount and size of DNA fragments. Initially, PARP activity was assessed in some samples using the published [^{32}P]NAD$^+$

incorporation PARP assay (15), but this assay lacked the required sensitivity for small clinical samples and an immunoblotting assay was developed from a published method (23) as described below.

Materials. All chemicals were obtained from Sigma (Dorset, United Kingdom) unless stated otherwise. Dulbecco's PBS was obtained from Life Technologies (Paisley, United Kingdom), sucrose, NaOH, and KCl were supplied by BDH (Lutterworth, United Kingdom) and digitonin by Boehringer Mannheim (Roche Diagnostics, Lewes, United Kingdom). The BCA protein assay kit (Pierce, Perbio Science, Rockford, IL) was used for protein concentration determinations. Milk powder was obtained from Marvel Premier Brands UK Ltd. (Spalding, United Kingdom), and Enhanced Chemiluminescence Western Blot Detection kits from Amersham (Little Chalfont, United Kingdom). The PARP inhibitors AG014699 and AG14361 were supplied by Pfizer GRD. Nycomed Lymphoprep was obtained from Axis-Shield (Oslo, Norway) and EDTA blood collection tubes from BD Vacutainer (Plymouth, United Kingdom). The 10H mouse monoclonal primary antibody was generously supplied by Prof. Alexander Bürkle (University of Konstanz, Germany), and the goat anti-mouse secondary antibody (horseradish peroxidase–conjugated) was obtained from DAKO (Ely, United Kingdom). The self-complimentary palindromic oligonucleotide (CGGAATTCCG) used to stimulate PARP activity was initially synthesized by Dr. J Lunec (Northern Institute for Cancer Research, Newcastle upon Tyne, United Kingdom), and subsequent supplies were obtained from Invitrogen (Glasgow, United Kingdom). Purified PAR polymer was obtained from Biomol Research Lab (Plymouth, PA).

Tissue culture of L1210 (quality control) cells. Cells were maintained in RPMI 1640 (Sigma) supplemented with 10% (v/v) FCS (Invitrogen) and 1 unit/mL penicillin-streptomycin solution (Sigma) in a Hereus incubator (Fisher Scientific, Manchester, United Kingdom) maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. L1210 cells used were obtained from American Type Culture Collection (Manassas, VA) and grown as a suspension to a density of ~ 6×10^5/mL at harvesting to ensure exponential growth. Aliquots of 1×10^6 cells for use as quality control samples were resuspended in 1 mL medium plus 10% (v/v) DMSO and 10% (v/v) FCS and frozen at −80°C. Replicate frozen samples were thawed and assayed at intervals up to 15 weeks to determine the potential deterioration of PARP activity with prolonged storage at −80°C.

Preparation of peripheral blood lymphocyte and tumor samples. Whole blood was collected into EDTA vacutainers and human PBLs were obtained by lymphopreparation according to the manufacturer's instructions. Tumor biopsies were collected from the operating theatre in a sterile container and placed immediately on ice. Within 30 minutes, tumor samples were snap frozen in liquid nitrogen and stored at −80°C until homogenized for analysis. The specimen was defrosted on ice and the wet weight was documented. The tissue was homogenized using a Pro 2000 instrument (Pro Scientific, Inc., Monroe, CT) in three isotonic buffer [7 mmol/L HEPES, 26 mmol/L KCl, 0.1 mmol/L dextran, 0.4 mmol/L EGTA, 0.5 mmol/L MgCl$_2$, 45 mmol/L sucrose (pH 7.8)]. For weights over 100 mg, homogenates were 1:4 (w/v; mg/μL). Smaller samples were homogenized to give final dilutions of 1:100 and 1:1,000. The homogenate was kept on ice throughout the process, and homogenization was done in 10-second bursts to prevent undue warming of the sample. Unless assayed on the day of homogenization, samples were refrozen to −80°C and stored at this temperature until analyzed. Before assay, the samples were further diluted with isotonic buffer where necessary to give a final dilution of 1:1,000.

Poly(ADP-ribose) polymerase assay procedure. Cell preparations was defrosted rapidly at room temperature and washed twice in ice-cold PBS. The cell pellets were resuspended in 0.15 mg/mL digitonin to a density of ~ 1×10^6 to 2×10^6 cells/mL for 5 minutes to permeabilize the cells (verified by trypan blue staining), following which 9 volumes of ice-cold isotonic buffer were added and the sample was placed on ice. Maximally stimulated PARP activity was measured in replicate samples of 20,000 cells in a reaction mixture containing 350 mmol/L NAD$^+$ and 10 mg/mL oligonucleotide in a reaction buffer of 100 mmol/L

Tris-HCl, 120 mmol/L $MgCl_2$ (pH 7.8) in a final volume of 100 μL as described previously (24) at 26°C in an oscillating water bath. The reaction was stopped after 6 minutes by the addition of excess PARP inhibitor (400 μL of 12.5 μmol/L AG014699) and the cells were blotted onto a nitrocellulose membrane (Hybond-N, Amersham) using a 24-well manifold. Purified PAR standards were loaded onto each membrane (0-25 pmol monomer equivalent) to generate a standard curve and allow quantification. Overnight incubation with the primary antibody (1:500 in PBS + 0.05% Tween 20 + 5% milk powder) at 4°C was followed by two washes in PBS-T (PBS + 0.05% Tween 20) and then incubation in secondary antibody (1:1,000 in PBS + 0.05% Tween 20 + 5% milk powder) for 1 hour at room temperature. The incubated membrane was washed frequently with PBS over the course of 1 hour and then exposed for 1 minute to enhanced chemiluminescence reaction solution as supplied by the manufacturer. Chemiluminesence detected during a 5-minute exposure was measured using a Fuji LAS3000 UV Illuminator (Raytek, Sheffield, United Kingdom) and digitized using the imaging software (Fuji LAS Image version 1.1, Raytek). The acquired image was analyzed using Aida Image Analyzer (version 3.28.001), and results were expressed in LAU/mm^2. Three background areas on the exposed blot were measured and the mean of the background signal from the membrane was subtracted from all results. The PAR polymer standard curve was analyzed using an unweighted one-site binding nonlinear regression model and unknowns read off the standard curve so generated. Results were then expressed relative to the number of cells loaded. Triplicate quality control samples of 5,000 L1210 cells were run with each assay, all samples from one patient being analyzed on the same blot.

Tumor homogenates were assayed in a similar manner; however, the homogenization process introduces sufficient DNA damage to maximally stimulate PARP activity and oligonucleotide was not therefore required. The protein concentration of the homogenate was measured using the BCA protein assay and Titertek Multiscan MCC/340 plate reader. Results were expressed in terms of pmol PAR formed/mg protein.

Patient treatment. Twelve patients with advanced malignant melanoma who had received no prior therapy for metastatic disease were studied, although previous radiotherapy or adjuvant therapy with biological agents was permitted. The trial was approved by Local Research Ethics Committees, and all patients gave written informed consent. Temozolomide was given at a dose of 200 mg/m²/d for days 1 to 5 of a 28-day cycle. Response to therapy was assessed every two cycles and patients were treated until progression of disease (appearance of new lesions or an increase in the sum of target lesion diameters by ≥25%). Inclusion criteria included age ≥18 years, histologically proven advanced metastatic melanoma, assessable disease, and adequate hematologic and biochemical variables. Patients with uncontrolled vomiting such as to make treatment with an oral agent impractical or those who received a biological therapy within 4 weeks of recruitment were excluded. In addition, subjects who were known to be pregnant or breast feeding, or HIV positive, were not entered. On day 1 of cycle 1 of treatment blood was taken before and 4 and 24 hours after treatment for analysis of temozolomide concentrations and PBL DNA damage, ATase levels, PARP activity assay, and DNA methylation. A more extensive pharmacokinetic sampling schedule was used for the first six patients recruited (before treatment, 30, 60, 120, 240, 360, and 1,480 minutes after treatment). Tumor biopsies were obtained under local anaesthetic from nine patients before and 4 or 24 hours after first temozolomide dose.

In addition to the patient studies, samples of whole blood (10 mL) were collected from a group of healthy volunteers at the same sampling intervals to allow an assessment of the variation in PARP activity over a 24-hour period in an untreated normal population.

Results

Patient demographics. Five female and 7 male patients were recruited; demographic data are shown in Table 1. The mean

Table 1. Patient demographics

	n
Mean age (range), y	57 (39-82)
Male/female	7/5
WHO performance status at entry (0/1/2)	4/6/0 (2 not stated)
Site of metastatic disease (%)	
Liver	8 (67)
Lung	5 (42)
Bone	1 (8)
Skin/lymph nodes	6 (50)
Brain	2 (17)
Previous therapy	
Adjuvant high-dose IFN	4
Adjuvant radiotherapy	1
Palliative radiotherapy	2
None	6

age of the patients was 57 years, and the predominant site of metastatic disease was in the liver, some patients having more than one site of metastatic disease. All patients undergone surgical excision of their primary tumor and subsequently developed metastatic disease. Previous treatment is documented in Table 1; no patient received prior chemotherapy. Four patients received adjuvant biological therapy and one patient received adjuvant axillary radiotherapy; two patients received radiotherapy in the palliative setting, one to whole brain following resection of an isolated brain metastasis and one to a painful bone lesion.

Clinical results. All patients completed their first cycle of treatment, allowing collection of all the planned pharmacokinetic and pharmacodynamic blood samples. Three patients were withdrawn after one cycle of treatment, two with early progressive disease, and one patient because he could not tolerate oral medication. Nine patients received at least two cycles of treatment (Table 2).

The treatment was well tolerated with no incidence of greater than Common Toxicity Criteria grade 1 nausea and vomiting. Fatigue was the most commonly reported adverse event but reached grade 2 in only two cases. One patient required a dose reduction for myelosuppression, with grade 2 neutropenia and grade 3 thrombocytopenia on the first cycle. Her second treatment was delayed by 10 days and she received a dose reduction to 150 mg/m²/d, as dictated by the protocol, subsequently receiving seven further cycles with no toxicity. One patient had a prolonged response to temozolomide and went on to receive a total of nine cycles of full-dose therapy without significant myelosuppression (i.e., two brief uncomplicated periods of grade 3 neutropenia during 9 months of treatment). All Common Toxicity Criteria grade 3 and greater toxicities are listed in Table 3, and these are in line with published data for temozolomide at this dose (25, 26).

Eleven of 12 patients were assessable for response. As described above, two individuals progressed through the first treatment cycle. Five patients showed progressive disease after two cycles and were withdrawn from treatment. A further two patients had disease stabilization after two cycles but progressive disease after 4 months of treatment. One patient had a prolonged response achieving a partial response and received

Table 2. Number of treatment cycles given

No. cycles	1	2	4	6	>6
No. patients	3	5	2	0	2

eight cycles of treatment, and one patient had a complete radiological response in her lung metastases with resolution of s.c. metastases. This patient was withdrawn from the study after nine cycles of treatment, having fractured through an isolated pelvic bone metastasis, to receive palliative radiotherapy.

Temozolomide pharmacokinetics. The first six patients recruited had more extensive pharmacokinetic sampling and concentration-time data are shown in Fig. 1. Limited pharmacokinetic sampling in subsequent patients showed that temozolomide was undetectable 24 hours after the first dose, consistent with the rate of drug clearance in the first six patients (Table 4). Furthermore, the values measured at 4 hours (mean $\pm$ SD, 3.0 $\pm$ 0.7 compared with 2.3 $\pm$ 0.6 µg/mL) were also consistent with those found in the patients with more extensive sampling.

DNA damage in peripheral blood lymphocytes. The alkaline COMET assay was used to assess the level of single-strand breaks present after a single dose of temozolomide. The results for the 10 patients whose samples generated data are shown in Fig. 2 and are the mean Olive moment at the three time points studied in each patient, each result being derived from the analysis of 50 cells. In 5 of 10 patients, an increase in DNA damage was observed 4 hours after dosing with temozolomide, levels returning to pretreatment or lower at 24 hours (i.e., before the next dose) in 3 of these 5 patients. In 7 of 12 patients, pretreatment and 4 hours post-treatment data for both PBL PARP activity and DNA damage were available (numbers 4-6 and 9-12). In 4 of 7 patients (i.e., patients 5, 6, 9, and 10), both PARP activity and DNA damage were elevated, and in patient 12, both DNA damage and PARP activity were reduced. Only in patients 4 and 11 was PARP activity clearly increased at 4 hours in the absence of DNA damage as measured by the COMET assay. The mean values for DNA damage for the entire patient group confirmed a general trend toward increased strand breaks at 4 hours (Table 4); however, due to interpatient variability, this increase was not statistically significant ($P = 0.15$, Student's t test).

O^6-alkylguanine alkyltransferase activity in peripheral blood lymphocytes and tumor biopsies. ATase activity was measured following temozolomide treatment in 10 patients (Table 4). ATase levels were reduced 4 hours after temozolomide treatment and remained below pretreatment levels at 24 hours. In only three patients were paired tumor biopsies available for analysis; a decrease in ATase levels was seen at 24 hours but not in the only tumor biopsy done 4 hours after receiving the first dose of temozolomide (data not shown).

DNA methylation. DNA methylation at the N^7 position of guanine in DNA was determined in PBLs from 10 patients using an immunoslot blot technique, suitable samples being unavailable from 2 subjects. In addition, methylation was analyzed in 11 tumor biopsies from 6 patients. N^7-guanine methylation was below the limit of quantification for all PBL samples before temozolomide administration (0.33 fmol N^7-guanine/µg DNA) Levels were detectable at 4 hours after dosing in all PBL samples, with levels being sustained over 24 hours (Table 4). Low

background levels of methylation were detected in all pretreatment tumor biopsies, 1.8 $\pm$ 0.7 fmol N^7/µg DNA ($n = 5$), but these were elevated >10-fold at 4 and 24 hours after the first dose of temozolomide [26 $\pm$ 11 ($n = 2$) and 23 $\pm$ 8 ($n = 4$) fmol N^7/g DNA, respectively]. The absolute levels of N^7-guanine methylation observed in PBL and tumor samples following temozolomide were similar (20-30 fmol N^7-guanine/µg DNA) although more variable in the latter case.

Poly(ADP-ribose) polymerase activity in peripheral blood lymphocytes in patients and healthy volunteers. Studies with L1210 cells frozen at $-80°C$ showed that there was no deterioration in PARP activity for at least 15 weeks (Fig. 3). All patient samples were analyzed <15 weeks after sampling. The mean baseline PARP activity in this patient population who have not previously received chemotherapy was 132 $\pm$ 84 pmol/10^6 cells (mean $\pm$ SD), with a range of 20 to 285 pmol/10^6 cells (Table 4). These values are similar to those observed in healthy volunteers where the mean PAR formation was 160 $\pm$ 140 (range, 15-615) pmol/10^6 cells. A significant increase in PARP activity was detected at 4 hours ($P = 0.033$, two-tailed Wilcoxon signed rank test), coincident with the increased temozolomide-induced DNA strand breakage. There was no statistical difference in PARP activity between the paired pretreatment and 24-hour samples. In PBL samples from healthy volunteers, there was no increase over baseline levels at 4 and 24 hours (Fig. 4A), suggesting that the increased activity seen at 4 hours in the patients was as a direct result of temozolomide administration and most likely associated with DNA strand breakage. These volunteer blood samples were analyzed using the [^{32}P]NAD$^+$ incorporation PARP assay, which tended to give lower estimates of PARP activity. Further, healthy volunteer studies showed no consistent variation in PBL PARP activity over 72 hours (Fig. 4B) measured using the immunoblot technique.

Poly(ADP-ribose) polymerase activity in tumors. PARP activity in the human tumor biopsies collected was very variable, consistent with the heterogenous nature of tumor material (Table 5). Notably, PARP activity was very low (1.7 pmol PAR monomer/mg protein) in the single melanotic melanoma sample collected (patient 3). When tumor PARP activity was expressed as a percentage of the pretreatment level, there was no consistent change in PARP activity at either 4 ($n = 2$) or 24 ($n = 4$) hours after temozolomide treatment, and comparison of the paired samples from each patient biopsied twice showed no significant difference in activity ($P \approx 1$, Wilcoxon signed rank test, two-tailed). The mean activity measured in pretreatment melanoma tumor biopsies (830 $\pm$ 600 pmol PAR monomer/mg protein, $n = 8$) was similar to that obtained

Table 3. Incidence of Common Toxicity Criteria grade 3 and 4 toxicities

Toxicity	No. patients with Common Toxicity Criteria grade 3/4 toxicity	% of all cycles given (total = 37)
Thrombocytopenia	1	3
Neutropenia	2	6
Headache (disease-related)	1	3
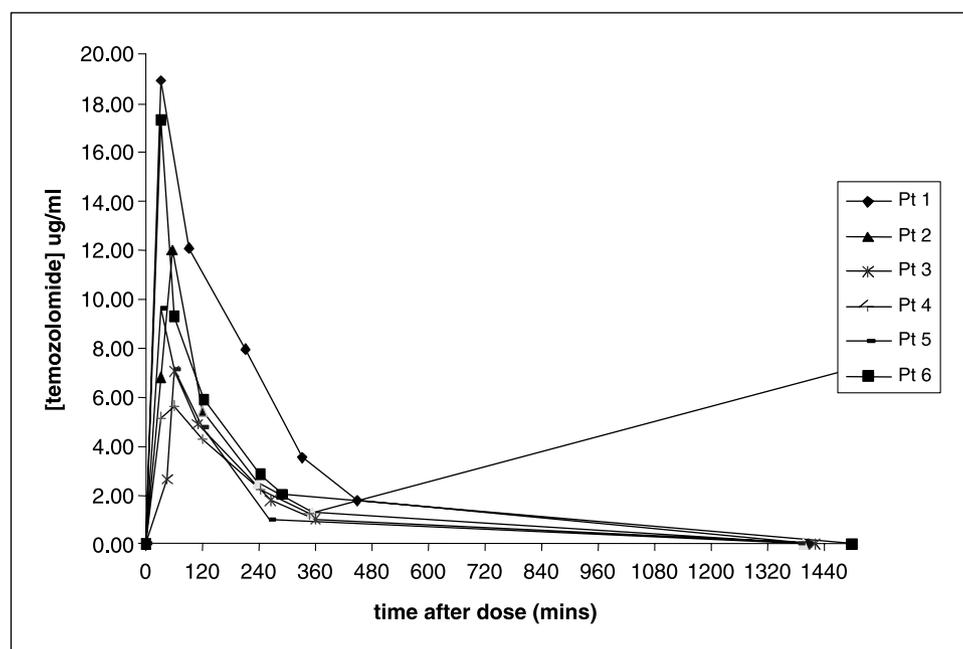
Fig. 1. Plasma temozolomide concentrations in patients 1 to 6 treated orally with 200 mg/m^2 temozolomide. Plasma temozolomide concentrations (μg/mL) measured over 24 hours after the first dose of temozolomide (200 mg/m^2) in the first six patients. Note: Patient 5 took the second dose of temozolomide before the 24-hour pharmacokinetic sample.

from homogenized untreated human colorectal tumor xenografts measured using a cross-validated radioactive PARP incorporation assay (1,299 ± 29 pmol NAD$^+$ incorporated/mg protein).[5] There was no clear relationship between the observed tumor PARP activity and N^7-guanine levels.

Discussion

This study was designed to investigate DNA damage (strand breaks and N^7-guanine methylation) and repair enzymes (ATase and PARP activity) in patients following treatment with temozolomide. The study also involved the validation of a PARP activity assay suitable for use on samples readily available in the clinical setting and provides a template for the evaluation of mechanism in trials involving the manipulation of DNA repair in combination with temozolomide.

The clinical efficacy and toxicity data obtained with temozolomide in this study are in line with those reported previously. The dose-limiting toxicity of temozolomide is myelosupression and other toxicities are mild. This was borne out in this study where only one patient required a dose reduction for myelosupression and treatment was otherwise well tolerated. The level of response observed (18% confirmed partial response rate) was similar to that published for the phase II and III trials of this drug in melanoma (25, 26), with several patients deriving clinical benefit from the treatment. The relatively high rate of early withdrawal from the study (after two or fewer cycles) reflects the poor prognosis of this disease rather than being a reflection of the toxicity of the treatment. Similarly, the pharmacokinetic results are consistent with the well-established profile of this compound (2, 27). All patients absorbed the oral preparation rapidly, and no subject showed prolonged retention of the drug in the plasma.

As with the clinical outcomes, our pharmacodynamic observations accord with previous experience in so far as this exists. The COMET assay analysis of DNA damage following temozolomide gave results consistent with data from an earlier study with dacarbazine (28). PBL ATase levels fell by ∼40% 4 hours after temozolomide therapy. Similar observations were made by Lee et al. (29) who found that when ATase levels were measured in PBLs from eight patients after dosing with temozolomide (150 mg/m^2) depletion of ATase was observed within 4 hours of the first dose with a median nadir of 53% of baseline 2 to 6 hours post-treatment, levels not returning to normal by 24 hours. This earlier study showed an 8-fold variation in pretreatment levels of ATase (69-593 fmol/mg protein); the units of measurement do not allow direct comparison with the values from the first eight patients in the current study, where pretreatment values ranged from 13 to 23 fmol/μg DNA. In the final four patients, data were also expressed in terms of fmol/mg protein and a similar range is observed to that published (81-535 fmol/mg protein). Previous studies have shown a relationship between the extent of PBL ATase depletion and pretreatment values in patients receiving temozolomide (29); however, in the study reported here, no such relationship was observed.

There are no published data in humans for the other variables we measured after temozolomide administration. As anticipated, methylation of DNA was detectable in PBLs at 4 and 24 hours following temozolomide. No difference in methylation level was detected between the two time points. A similar pattern was observed in the tumor biopsy specimens. Interestingly, N^7 methylation was detected in all baseline tumor biopsies but not in PBLs before treatment. It is unclear whether this is due to the nature of the antibody used to probe for the methylated base or whether it truly reflects "tolerated" endogenous DNA damage within the tumor. No patient previously received a methylating agent.

PARP activity was measured in PBLs from all patients and in tumor samples in 9 of 12 patients. Both the absolute level and

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[5] E.R. Plummer, unpublished results.

Table 4. Summary of temozolomide pharmacokinetic analysis and DNA damage assessment in PBLs of all patients after first dose of temozolomide

| Patient	Plasma (temozolomide; μg/mL)			ATase (fmol/μg DNA)			*N*7-methylguanine (fmol/μg DNA)			Olive moment			PARP activity (pmol PAR/10^6 cells)		
	0	4 h	24 h	0	4 h	24 h	0	4 h	24 h	0	4 h	24 h	0	4 h	24 h
1	0	3.5	0	19.9	11.5	16.8	0	16.4	25.6	20.1	23.3	10.4	—	—	—
2	0	2.5	0	18.0	10.9	11.3	0	18.9	24.3	19.2	18.9	26.2	—	—	—
3	0	1.8	0	12.6	14.3	11.0	—	19.7	15.6	8.1	23.5	10.2	—	—	—
4	0	2.3	(7.8)	22.8	17.7	15.5	0	13.9	22.8	19.8	16.4	21.2	20	130	10
5	0	1.0	0	20.7	9.0	4.6	0	21.6	20.1	5.8	14.4	12.7	135	695	195
6	0	2.8	0	13.8	4.8	5.3	0	28.3	21.3	8.7	21.1	7.0	20	100	15
7	0	3.7	0	—	—	—	—	—	—	—	—	—	90	75	20
8	0	3.7	0	—	—	—	—	—	—	—	—	—	165	225	70
9	0	2.1	0	13.0	9.8	9.9	0	23.5	19.1	8.9	12.9	6.9	170	205	275
10	0	2.7	0	17.7	42.6	16.8	0	—	—	1.3	3.7	4.7	285	555	500
11	—	—	—	12.2	9.9	10.0	0	19.1	24.1	3.6	3.5	5.9	185	1160	100
12	0	2.6	0	32.5	11.6	—	0	—	—	26.4	19.3	17.6	120	80	—
Mean	0	2.6	0	18.3	14.2	11.2	0	20.2	21.6	12.2	15.7	12.3	132	358	148
SD	0	0.8	0	6.2	10.5	4.5	0	4.4	3.3	8.5	7.3	7.2	83	373	171

variation in pretreatment PBL PARP activity in this untreated patient population was similar to that observed in healthy volunteers. This is the first documentation of the measurement of PARP activity in patients receiving chemotherapy and clearly shows that PARP activity in PBLs was significantly increased at 4 hours after a single dose of temozolomide. Indeed, 4 hours after temozolomide administration, only one patient showed >20% reduction in PARP activity, and in this patient, DNA strand breaks were also apparently reduced. Temozolomide pharmacokinetics were unremarkable in this patient. These data suggest that the temozolomide-induced activation of PARP is related to DNA strand break induction.

PARP-1 is reported to be abundant and constitutively expressed in human cell cultures with an average of one copy per 1,000 bp (9). Following activation by DNA single-strand breaks, there is rapid polymer synthesis and automodification of PARP-1 followed by PAR glycohydrolase-mediated degradation of polymer and recycling of the enzyme. The half-life of PAR is in the order of minutes (13). Given the high endogenous expression of PARP-1 and this dynamic equilibrium, further enzyme synthesis in response to DNA damage would not be expected. However, PARP may not be constitutively expressed at maximum levels in normal tissues; indeed, the activity in human PBLs was ~25% of that measured in L1210 cells (data not shown) and increased expression of the enzyme in response to DNA damage is possible. Alternatively, the increase in PARP
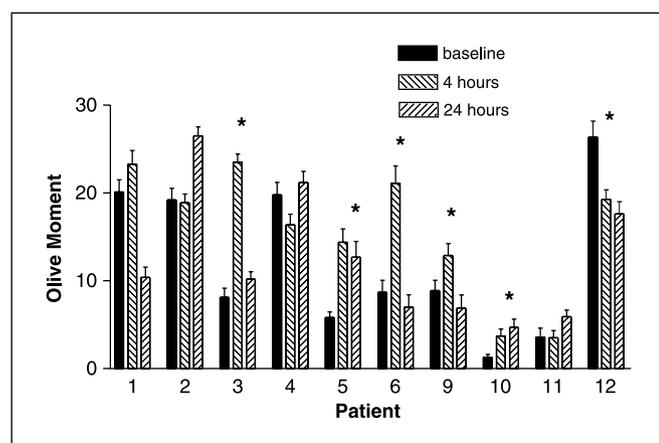
Fig. 2. DNA damage (Olive tail moments) in PBLs before and 4 and 24 hours after 200 mg/m² temozolomide. DNA damage (single-strand breaks) in PBLs after the first dose of temozolomide (200 mg/m²) measured using the alkaline COMET assay. *Columns*, mean for 50 COMETs measured in each sample; *bars*, SE. Data are from 10 patients; samples from patients 7 and 8 were unsuitable for analysis. *, $P \leq 0.05$, significant difference between baseline and 4-hour values (unpaired, two-tailed *t* test).
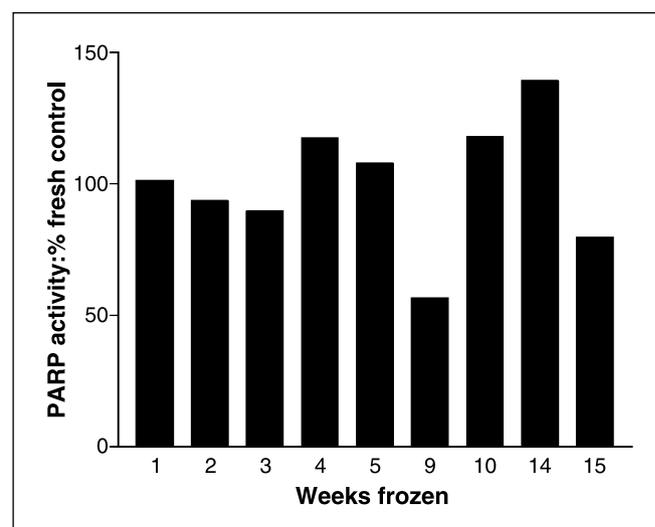
Fig. 3. Variation in PARP activity in L1210 cells after storage at −80°C for up to 3 months. PARP activity in L1210 cells examining the effects of storage of samples over a 3-month period. Data from paired L1210 cell samples, fresh and frozen assayed in triplicate on the same day using NAD⁺ incorporation PARP activity assay. Results displayed as control data expressed as a percentage of the fresh control value assayed on that day. No deterioration observed over the period studied.
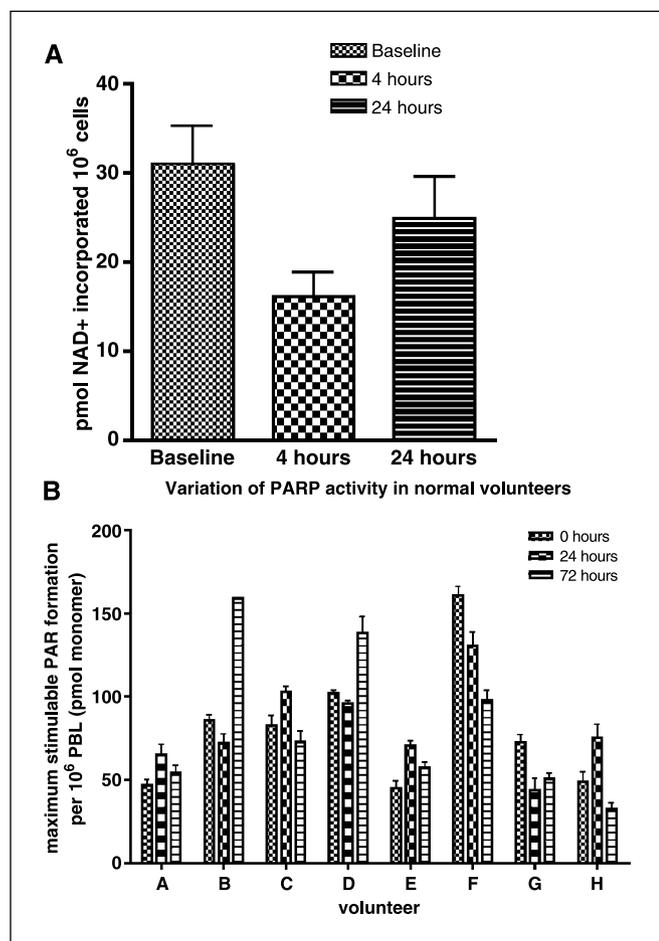
Fig. 4. Variation in maximally stimulated PAR formation over time in PBL samples from healthy volunteers. *A*, maximally stimulated PARP activity in serial blood samples from healthy volunteers drawn at baseline and 4 or 24 hours later. *Columns*, mean of samples analyzed in triplicate, *n* = 14 (baseline), 7 (4 hours), and 8 (24 hours); *bars*, SE. Values were determined using ^{32}[P]NAD$^+$ incorporation assay, which gives lower estimates of PARP activity than immunoblot assay. No significant variation in PARP activity observed over one 24-hour period. *B*, maximally stimulated PARP activity in serial blood samples from healthy volunteers drawn at baseline and at 24 and 72 hours. *Columns*, mean of triplicate samples from eight normal healthy volunteers; *bars*, SE. No variation maximally stimulated PARP activity, measured as ability to form PAR using immunoblot assay, was observed.

activity observed at 4 hours could be due to temozolomide-induced release of sequestered PARP-1 or enzyme activation following post-translational modification.

In the ongoing first-in-human trial of a PARP inhibitor in combination with temozolomide, a primary end point of the phase I study is proof of PARP inhibition in both PBLs and the target tissue. For ethical reasons, a combination study with temozolomide is being done rather than a PARP inhibitor alone study. Hence, it was important in the current study to show that temozolomide on its own did not reduce PARP activity and potentially confound the interpretation of results from the combination trial. As described above, PARP activity was *increased* by temozolomide treatment, allowing confidence that any reduction in activity observed in the PARP inhibitor clinical trial is due to the PARP inhibitor and not the temozolomide. Preclinical evidence shows that inhibition of PARP and hence base excision repair following temozolomide treatment increases peak levels of strand breaks and the persistence of *N*-methyl purines in DNA (30).

Although PBLs can be a useful surrogate tissue for investigating systemic pharmacodynamics, it is important to measure target tissue effects as well, and PARP activity was measured in human tumor samples in the current study. There was wide variation in PARP activity detected in the tumor biopsies, potentially reflecting the heterogeneity of human tumors. Preclinical data in human tumor xenografts indicated that there were high levels of PARP activity in tumors when compared with mouse liver (19), and consistent with this result, data from human liver biopsies show lower levels in normal liver compared with hepatocellular cancer samples (31). Similar results have been observed comparing normal colonic epithelium and malignant polyps (32). The majority of the melanoma biopsies obtained in the current study showed comparatively high levels of PARP enzyme activity, relative to the activity in PBLs from the same patient, and if this high PARP activity contributes to tumor resistance to temozolomide, a PARP inhibitor in combination with temozolomide may increase antitumor effects.

Four patients demonstrated clinical benefit with disease stabilization for 4 months or longer. Two of these patients had low pretreatment ATase levels (13 and 12 fmol/μg DNA) and

Table 5. PARP activity in tumors of patients treated with 200 mg/m^2 temozolomide

Patient	PARP activity (pmol PAR formed/mg protein)			Post-treatment PARP activity as a % of pretreatment		
	Baseline	4 h	24 h	Baseline	4 h	24 h
1	463	—	496	100		109
2	ND*	2.5	—	—	—	
3†	1.7	—	18	—	—	—
4	2754	—	29	100		1.1
5	819	2926	—	100	376	—
6	606	—	2875	100		475
7	1311	—	259	100		20
8	3609	2059	—	100	57	
9	3596	—	No biopsy taken	100	—	No biopsy taken

*The pretreatment biopsy from patient 2 was too small to homogenize.
†Patient 3 had a melanotic tumor; all others were amelanotic.

one had a high level (18 fmol/μg DNA); no data are available for the patient who had a prolonged response due to loss of the sample during transport. The two patients who had early progression of disease (after one cycle of treatment) had normal or high initial ATase levels (21 and 32.5 fmol/μg DNA, respectively) and high pretreatment PARP activity (135 and 120 pmol PAR/10^6 cells). A correlation has been shown previously between high tumor ATase activity and poor response to temozolomide in patients with malignant glioma (33), but no relationship was observed in patients with metastatic melanoma (34).

In conclusion, we report here for the first time an in-depth evaluation of temozolomide pharmacodynamics by measuring its effect on a battery of DNA damage and repair variables. Furthermore, this study allowed the validation of an immunoblot assay for measuring PBL and tumor PARP activity in clinical samples essential for the pharmacodynamic end point in the PARP inhibitor clinical trial. The observed increase in PARP activity after dosing with temozolomide provides confidence that PARP inhibition can be used as a direct pharmacodynamic marker after the administration of the combination of temozolomide and a PARP inhibitor. The changes in ATase activity and DNA damage observed following standard temozolomide dosing are consistent with the published results, confirming that the study population reported here are typical of the general patient population and provide important comparative data for the interpretation of the outcome of temozolomide/PARP inhibitor combination therapy.

Acknowledgments

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Temozolomide Pharmacodynamics in Patients with Metastatic Melanoma: DNA Damage and Activity of Repair Enzymes O^6-Alkylguanine Alkyltransferase and Poly(ADP-Ribose) Polymerase-1

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