

Temozolomide: The Effect of Once- and Twice-a-Day Dosing on Tumor Tissue Levels of the DNA Repair Protein *O*⁶-Alkylguanine-DNA-Alkyltransferase¹

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ABSTRACT

Temozolomide (TMZ) is a methylating agent of the imidotetrazine class, whose cytotoxic product is *O*⁶-methylguanine DNA adducts, which initiate a futile recycling of the mismatch repair pathway causing DNA strand breaks and apoptotic cell death in mismatch repair proficient cells. The DNA repair protein *O*⁶-alkylguanine DNA alkyltransferase (AGT) repairs these adducts in a suicide manner and reduces the cytotoxic action of TMZ. An antitumor threshold is reached when sufficient adducts are formed by TMZ to inactivate AGT. In this study, we evaluated the relation between TMZ dosing and AGT depletion in patients with deep visceral tumors and in peripheral blood mononuclear cells (PBMCs) to determine whether the dose of TMZ was sufficient to inactivate AGT and lead to therapeutic efficacy. To do so, we compared single dose therapy with a novel twice daily regimen in a laboratory correlate-driven Phase I dose escalation study. p.o. bolus dose TMZ 200 mg/m² daily times five was compared with the same bolus on day 1 followed by nine doses at 12-h intervals of 50, 75, 90, or 100 mg/m². Dose-limiting toxicity in the bid regimen (grade IV thrombocytopenia and neutropenia) was seen at 100 mg/m², cumulative dose 1100 mg/m², and the maximum tolerated dose was 1010 mg/m². The degree of tumor tissue AGT activity depletion measured in biopsies before and on day 5 of therapy varied widely, between 0 (in 3 patients) and 99% (in 1), with the majority of patients (10 of 15) having 52–84% tumor AGT depletion. In contrast, AGT activity in PBMCs fell rapidly during TMZ administration to undetect-

able levels in all dosage groups on day 5 but did not correlate with tumor AGT depletion. TMZ pharmacokinetics were dose proportional; no accumulation occurred >5-day period in the bid regimen. Two partial responses were seen, lasting 3 and 4 months. Five additional patients achieved prolonged stabilization of disease for 4–6 monthly cycles. This is the first study to document that at maximum tolerated doses, TMZ depletes PBMC AGT but only partially and variably depletes visceral tumor AGT in most patients, even during twice daily dosing. Drug combinations or schedules designed to maximally deplete tumor AGT might improve TMZ efficacy.

INTRODUCTION

TMZ³ (3,4-dihydro-3-methyl-4-oxoimidazo-[5,1-d]-1,2,3,5-tetrazin-8-carboxamide), a 3-methyl analogue of mitozolomide, is a methylating agent of the imidotetrazine class. In common with DTIC, its active metabolite is the linear triazine MTIC. Whereas DTIC requires initial activation in the liver, TMZ is metabolized to MTIC in the peripheral blood at physiological pH. *In vitro* and human xenograft studies have shown a broad spectrum of activity of TMZ in murine tumors (1–3). Increased survival times were observed in solid tumor models (M5076 reticulum cell carcinoma, B16 melanoma, C26 adenocarcinoma, and Lewis lung carcinoma) and in hematological models (ADJ/PC6A plasmacytoma, TLX5 lymphoma, L1210 leukemia, and P388 leukemia) using a 5-day p.o. or i.p. dosing schedule (2).

Only 8% of the alkylation attributable to TMZ occurs at the *O*⁶ position of guanine, where it primarily forms *O*⁶-methylguanine (*O*⁶mG) DNA adducts; this is the main cytotoxic target for this drug (4). Whereas 70% of the alkylation attributable to TMZ occurs at the *N*⁷ position of guanine, the *N*⁷mG adduct is much less toxic (4, 5). During DNA synthesis, *O*⁶-mG preferentially mispairs with thymine resulting in the *O*⁶-mG:T bp (6). Most of the cytotoxicity induced by the *O*⁶-mG adducts seem to be associated with aberrant DNA repair attributable to abortive mismatch repair of both the *O*⁶-mG:C and *O*⁶-mG:T bp. The *O*⁶-mG:T bp mismatch is recognized by the mismatch repair system with removal of the T opposite the *O*⁶-mG adduct, but removal of the *O*⁶-mG does not take place. Thus, a repetitive cycle of mismatch repair recognition and processing to be placed into regeneration of the mismatch occurs. This futile recycling of the mismatch repair pathway ultimately results in

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³ The abbreviations used are: TMZ, temozolomide; AGT, *O*⁶-alkylguanine DNA alkyltransferase; PBMC, peripheral blood mononuclear cell; MTIC, monomethyl triazenoimidazole carboxamide; DTIC, dacarbazine; DLT, dose-limiting toxicity; MTD, maximum tolerated dose; BG, *O*⁶-benzylguanine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CT, computed tomography; AUC, area under the curve; PR, partial remission.

sister chromatid exchanges and DNA strand breaks, activation and caspase-mediated cleavage of poly (ADP-ribose) polymerase, and apoptotic cell death.

AGT is a DNA repair protein that repairs adducts at the O^6 position of guanine. Each AGT molecule removes one adduct through covalent binding of the alkyl group to the cysteine residue at amino acid number 145. During this process, irreversible inactivation of the protein occurs, and synthesis of new molecules is required to regenerate AGT activity (7, 8). The natural targets for AGT are small alkyl groups including methyl, ethyl, isopropyl, hydroxymethyl, and chloroethyl adducts. Repair of TMZ-induced O^6 mG adducts by AGT prevents cytotoxicity. Depletion of alkyltransferase activity enhances the cytotoxicity of TMZ (8); however, an intact mismatch repair system is required to achieve its cytotoxic effect (9). *In vitro* data show that persistence of O^6 -MG adducts and their accumulation over time correlate with depletion of AGT activity and contribute significantly to the cytotoxicity of TMZ (10).

Preclinical studies in rats and dogs have shown absolute p.o. bioavailability of TMZ of 96–100% compared with i.v. administration. The drug was rapidly eliminated from plasma with a $t_{1/2}$ of 1.2 h after either p.o. or i.v. administration. The drug penetrated rapidly into the brain with the extent of penetration ranging between 35 and 39% on the basis of the brain: plasma ratio (data on file at Schering Plough Research Institute).

Alkylating agents, by virtue of their mechanism of action, cause depletion of AGT. AGT depletion with O^6 -mG has been demonstrated, but depletion of AGT was incomplete (11). O^6 -mG combined with BCNU was unsuccessful in increasing the therapeutic index of BCNU in human tumor xenografts (12). A clinical trial using streptozotocin as an AGT-depleting agent demonstrated incomplete inactivation of AGT, and the streptozotocin/BCNU combination caused severe hematological toxicity (13). More recently, we showed the capacity of BG to completely deplete AGT activity in human tumors.

In Phase I clinical trials with TMZ, single and multiple dose pharmacokinetics with a once-a-day schedule have been well defined over a range of 100–1000 mg/m² doses (14, 15). The drug has a half-life of 1.8 h, the C_{max} and AUC are dose proportional, and the clearance and volume of distribution are dose independent. Interindividual variability was small. No accumulation was observed after multiple doses. Observed DLT was myelosuppression, neutropenia, and/or thrombocytopenia (14). The efficacy of TMZ is well established in Phase I/II clinical trials. In most studies, a once daily for 5 days p.o. regimen has been used (16). Efficacy has been particularly demonstrated in high-grade gliomas and metastatic malignant melanoma (17, 18). Whereas TMZ has been shown in laboratory studies to deplete AGT, its activity in humans within the therapeutic dose range is unknown. In addition, once daily administration appears to allow the regeneration of AGT activity, thereby reducing its antitumor efficacy.

The latter studies used AGT immunohistochemistry as the marker for AGT activity (18). AGT protein by Western blot analysis is known to persist after AGT inactivation and can make immunohistochemical interpretations unreliable (19). A highly specific method for the biochemical measurement of AGT activity is available in our laboratory and appears to be a reliable indicator of functional AGT activity (20). Because

preclinical data strongly suggest that methylating agent efficacy is dependent on AGT depletion, we designed a clinical trial to examine AGT depletion in human tumors and in PBMCs. Because the $t_{1/2}$ of TMZ is short, we hypothesized that optimal depletion of AGT activity might best be achieved with a more frequent dosing schedule. To define the optimal dosing schedule for this drug, we used a twice-a-day dosing schedule to examine the pharmacokinetic/pharmacodynamic relationship of TMZ plasma concentrations with AGT activity (using a biochemical assay) in PBMCs and tumor tissue.

MATERIALS AND METHODS

Study Objectives. The primary objective of this clinical trial was to characterize the safety profile, DLT, and MTD of TMZ given in a twice daily dosing schedule >5 consecutive days. Secondary objectives included evaluation of the biochemical modulatory effect of TMZ on AGT activity in tumor tissue biopsies and in PBMCs, profiling of plasma pharmacokinetics, and preliminary clinical assessment of antitumor activity.

Patient Selection. Patients enrolled in this study were males or females ≥ 18 years of age with metastatic solid tumors, a performance status of ECOG 2 or better (life expectancy > 12 weeks), and an adequate renal (creatinine < 1.6) and hepatic function (total bilirubin within normal range; aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase < twice upper limit of normal). All patients were required to have clinically or radiographically measurable lesions, accessible to biopsy. Institutional Review Board approval and patient informed consent were obtained for all patients. A separate informed consent was obtained for pretreatment and posttreatment CT-guided tumor tissue biopsies.

Phase I Clinical Trial Design. On day 1/cycle 1, patients received a p.o. bolus dose of 200 mg/m². This was followed by nine further p.o. doses given at 12-h intervals. The starting dose was 50 mg/m²/dose (total dose >5 days of 650 mg/m²). The dose of TMZ was increased in cohorts of 3 patients with dose escalation to 75 mg/m²/dose (total dose >5 days of 875 mg/m²) and 100 mg/m²/dose (total dose >5 days of 1100 mg/m²). After the 100 mg/m² dose was established as the DLT, a dose reduction to 90 mg/m²/dose (total dose >5 days of 1010 mg/m²) was performed to establish the MTD. An additional cohort of 8 patients was treated with a p.o. dose of 200 mg/m² daily for 5 days. Cycles were repeated at 28-day intervals.

Plasma concentrations of TMZ were assessed >6-h period on days 1, 2, and 5. Plasma samples were drawn before treatment and at 0.5, 1, 2, 4, and 6 h after the morning dose. On days 3 and 4, plasma samples were drawn at baseline and at 4 h after dosing. Whole blood samples for the measurement of AGT activity in PBMCs were drawn at baseline and 4 h after drug administration daily and additionally at the 2-h time point on days 1, 2, and 5. To study differences in the pharmacokinetic/pharmacodynamic actions of once and twice daily TMZ administration, tumor tissue biopsies were performed on 15 patients before therapy (≤ 5 days) and repeated 2 h after dosing on day 5. The technique for handling of biopsies has been described previously (14).

Tumor Tissue Biopsies. Clinical tumor biopsies (>200) have been performed to date at our institution, and this tech-

nique has proven to be safe. The solid tumor samples were obtained by CT-guided 20-gauge Chiba cutting needle biopsies and immediately frozen in liquid nitrogen. To divide the samples, the tumor was placed on a metal pan over liquid nitrogen vapors and divided into 2-mm segments for histology and 4–5-mm segments for AGT assay. Each sample for AGT assay contained 5–20 mg of tissue (wet weight) and 0.4–1 mg of protein. Multiple sections were assayed for AGT from each sample. The AGT assay requires use of as little as 2–5 mg of tissue wet weight. Tumor biopsies contain mostly tumors, but they may contain areas of necrosis, fibrosis, or normal tissue. Pre- and posttreatment values represent mean AGT activity (duplicate assays) in between two to four segments of the biopsy. To be evaluable, the tumor must be present in both pre- and posttreatment biopsies (20, 21).

Assay of Alkyltransferase Activity in Tumor Tissue Biopsies and PBMCs. The activity of alkyltransferase in tumor tissue extracts was measured as a removal of the [³H]methyl adduct from the *O*⁶ position of guanine in DNA by incubating cell or tissue extracts with DNA substrate as described previously (20). Briefly, substrate [³H]methyl-DNA was prepared by reacting calf thymus DNA with [³H]methylnitrosourea (specific activity was 0.06 fmol/dpm, corrected for decay of [³H]). New substrate was also directly compared with older substrate so that the reporting of AGT activity from this laboratory has remained constant over the past 15 years. The specific activity was confirmed by establishing a standard curve using calf thymus DNA as a negative control and a NIH3T3-*MGMT* transfectant cell extract as a constant substrate. During method development, we determined that removal of *O*⁶mG is linear with respect to *N*⁷mG when increasing amounts of AGT containing cell extract is added in the range of 12–85% removal of *O*⁶mG. All assays were performed within this range.

Preparation of Tumor Tissue Extracts. Each tumor segment was placed in 250 μ l of cell extract buffer [cell extract buffer containing 70 mM HEPES (pH 7.8), 0.1 mM EDTA, 5% glycerol, 1 mM DTT, and 25 μ M spermidine] and sonicated three times for 10 s at 4°C to complete cell disruption using a microsonicator equipped with a 3/32" diameter probe (Branson Ultrasonics, Corp., Danbury, CT). Aliquots (20 μ l) for DNA quantification by Hoescht dye stain were removed, and tumor tissue extracts were centrifuged at 13,000 rpm for 2 min to remove cellular debris. Protein concentration was determined by Bio-Rad protein assay. The sample was frozen at –80°C or processed immediately for AGT assay.

Enzyme Assay. Protein (50–250 μ g) of tumor tissue extract was incubated with [³H]methyl DNA in CEB buffer in the total volume of 300 μ l for 60 min at 37°C. The reaction mixture containing excess substrate DNA, such as total AGT activity, could be determined. The reaction was stopped with 7.5% trichloroacetic acid at 4°C for 30 min. The precipitate was collected by centrifugation at 13,000 rpm for 2 min and washed with 300 μ l of 80% ethanol. Methylated purines were liberated from precipitated DNA during hydrolysis with 150 μ l of 0.1 N HCl at 80°C for 1 h. [³H]*O*⁶-methylguanine and [³H]*N*⁷-methylguanine, which was constant during incubation and served as the internal standard, in supernatant were separated by reverse-phase high-pressure liquid chromatography and quantitated by liquid scintillation counting. Radioactivity (dpm) in each peak

(*N*⁷mG and *N*⁶mG) derived from the substrate ranged from 350–800 dpm for the *O*⁶mG peak (21–48 fmol) and 12-fold higher values for *N*⁷mG. Peak values were corrected for background radioactivity on the basis of both the positive and negative controls. AGT activity was expressed as fmol *O*⁶methylguanine removed/ μ g DNA. Duplicate assays were performed on each biopsy segment. The limit of detection is on the basis of the linear portion of the assay and was defined as AGT activity (fmol/ μ g DNA) corresponding to 12% of the *O*⁶mG present in the substrate [³H]methyl DNA used in each assay. For tumor biopsy samples, the limit of detection value was 0.1 fmol/ μ g sample DNA.

PBMCs from whole blood samples were obtained by Ficoll-Hypaque separation and assayed for AGT as described above. For PBMCs, the limit of detection value was 0.05 fmol/ μ g sample DNA and is lower for tumor samples because more cell extract, and thus more [³H]DNA substrate, is used in each assay (20).

TMZ Assays in Plasma. Blood samples for TMZ assays were collected in prechilled syringes, placed in chilled heparin tubes, and immediately cooled in an ice water bath. Plasma was separated \leq 30 min at 3000 rpm in a refrigerated centrifuge maintained at 4°C. Immediately after centrifugation, a 2-ml volume of plasma was transferred to a plastic tube containing 0.1 ml of 8.5% phosphoric acid. Tubes were capped, frozen, and stored at –20°C. Assays were performed by Schering-Plough Research Institute as described previously (22).

Preclinical Assessment of TMZ-mediated Depletion of AGT Activity and Cytotoxicity. SW480 human colon cancer cells were exposed to TMZ at increasing concentrations from 0–500 μ M for 2 h. Cells were washed and assayed for AGT as above. To determine cytotoxicity, cells were exposed to TMZ at concentrations of 0–1500 μ M for 2 h. Cells were assayed for AGT activity as described above.

RESULTS

Preclinical Assessment of TMZ. TMZ depleted AGT activity in SW480 cells (baseline 6.7 ± 1.3 fmol/ μ g DNA) in a dose-dependent fashion. The ED₅₀ was 58 μ M (11 μ g/ml), and the ED₉₀ was 190 μ M (36 μ g/ml; Fig. 1A). In cytotoxicity experiments, clonogenic survival of SW480 cells was reduced in a dose-dependent fashion, with an IC₅₀ of 370 μ M (71 μ g/ml) and an IC₉₀ of 900 μ M (180 μ g/ml; Fig. 1B). Thus, depletion of AGT was a prerequisite for optimal cytotoxic effect of TMZ.

Clinical Trial. A total of 22 patients were treated with TMZ (14 females and 8 males). The mean age was 59 (range, 35–84) years. Twelve patients had metastatic colorectal carcinomas, and the remaining patients had metastatic carcinoma of the lung ($n = 2$), breast cancer ($n = 2$), carcinoma of unknown primary ($n = 2$), pancreatic carcinoma ($n = 2$), leiomyosarcoma ($n = 1$), gastric cancer ($n = 1$), and carcinoma of the sinus ($n = 1$; Table 1).

Clinical Tolerance and Safety Data. Fourteen patients enrolled in this study received twice daily dosing of TMZ for 5 days with cumulative TMZ doses ranging from 650 mg/m² to 1100 mg/m² in cohorts of 3 patients (Table 1). DLT was seen at 1100 mg/m²; in patient 7, Grade III thrombocytopenia was seen on day 29, and in patient 8, Grade IV thrombocytopenia oc-

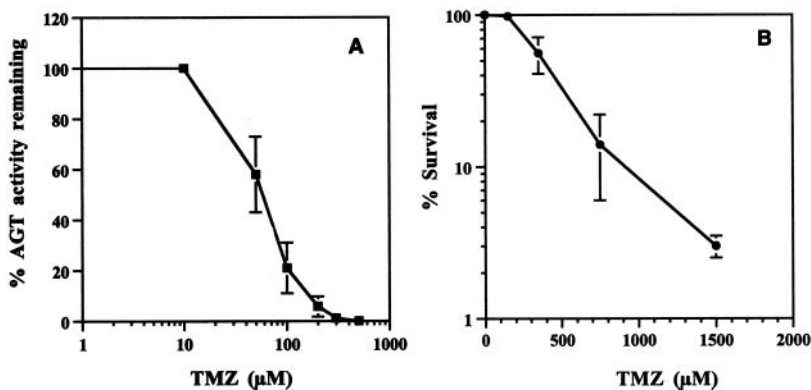


Fig. 1 A, AGT depletion in the human colon cancer cell line SW480 after treatment with 10–500 µM TMZ for 2 h. SW480 cells expressed high levels of AGT (6.7 ± 1.3 fmol/ µ g DNA). TMZ depleted AGT activity because the repair of O⁶-mG DNA adducts resulted in an EC₅₀ of 58 µM and EC₉₀ of 190 µM. B, cytotoxicity of TMZ in the human colon cancer cell line SW480. Cells were treated with 0–1500 µM TMZ for 2 h. Clonogenic survival showed that the IC₅₀ for TMZ was 370 µM, and IC₉₀ was 900 µM.

Table 1 Patient characteristics

Patient no.	Age	Gender	Tumor primary	Biopsy site ^a	Dosage group (mg/m ²)	Cycles of TMZ
1	70	Male	Lung		650	2
2	67	Female	Colon		650	5
3	68	Female	Gastric		650	2
4	60	Female	Lung		875	2
5	84	Male	Colon		875	6
6	51	Female	Colon		875	5 PR
7	35	Male	Sinus	Liver	1100	4
8	63	Female	Colon	Liver	1100	2
9	52	Female	Pancreas	Liver	1010	1
10	59	Female	Colon	Liver	1010	2
11	66	Female	Colon	Liver	1010	4
12	36	Female	Unknown 1°	Liver	1010	2
13	59	Female	Colon		1010	6
14	46	Female	Colon	Liver	1010	1
20	65	Male	Rectal	Liver	1000	3
21	58	Female	Colon	Liver	1000	2
22	58	Female	Breast	Liver	1000	2
23	58	Male	Pancreas	Liver	1000	2
24	57	Male	Melanoma	Lymph node	1000	5 PR
25	69	Female	Breast	Liver	1000	1
26	49	Male	Intestine	Liver	1000	2
27	69	Male	Colon	Liver	1000	2

^a Biopsies not done on patients 1–6 and thereafter were performed 2 h after the TMZ dose on day 5.

occurred on day 23, and Grade III neutropenia was observed on day 29. A dose reduction was made to 1010 mg/m², and a further 6 patients were treated, and no hematological toxicity (Grade III or greater) was observed, establishing this as the MTD. Lymphocytopenia, observed at all dose levels, was transient and did not result in clinical sequelae.

An additional cohort of 8 patients (5 males and 3 females) received a single daily dose of 200 mg/m² of TMZ daily for 5 days (cumulative dose 1000 mg/m²). These patients were not part of the MTD dose titration; however, we compared the toxicity, AGT depletion, and pharmacokinetics for these patients with the twice-a-day regimen. Patient 22 developed Grade III thrombocytopenia on day 26 of cycle 1. Otherwise, no DLT was observed. Transient lymphocytopenia without clinical sequelae was similarly observed in this patient group.

Depletion of AGT Activity in PBMCs. The advantage of AGT monitoring in PBMCs is that dynamics of drug effect can be evaluated, although it may not predict AGT depletion in

tumors. Mean AGT activity in PBMCs is shown in Fig. 2. At baseline, mean AGT activity was 7.38, 11.21, 10.1, and 7.56 fmol/µg DNA in the 650, 875, 1010, and 1100 mg/m² groups, respectively. AGT activity declined rapidly within the first 4 h. Perhaps because patients in all four dosage groups received the same initial bolus dose of TMZ (200 mg/m²), the different 12-h doses resulted in a similar degree of AGT inactivation at 24 h, before the third dose (84, 88, 81, and 94%, respectively). PBMC AGT activity remained low in all treatment groups over the 5-day treatment. Four h after dosing on day 5, the percentage of AGT inactivation was 95, 98, 97, and 96% for 650, 875, 1010, and 1100 mg/m² groups, respectively. A definite dose response was not seen. Notably, at 24 h, patients receiving single-dose TMZ had a mean of 50% baseline AGT activity, whereas patients receiving the twice daily dose regimen had <10% baseline AGT activity in PBMCs (*P* < 0.0001). Thus, in PBMCs, more rapid AGT depletion occurs with the bid dose schedule.

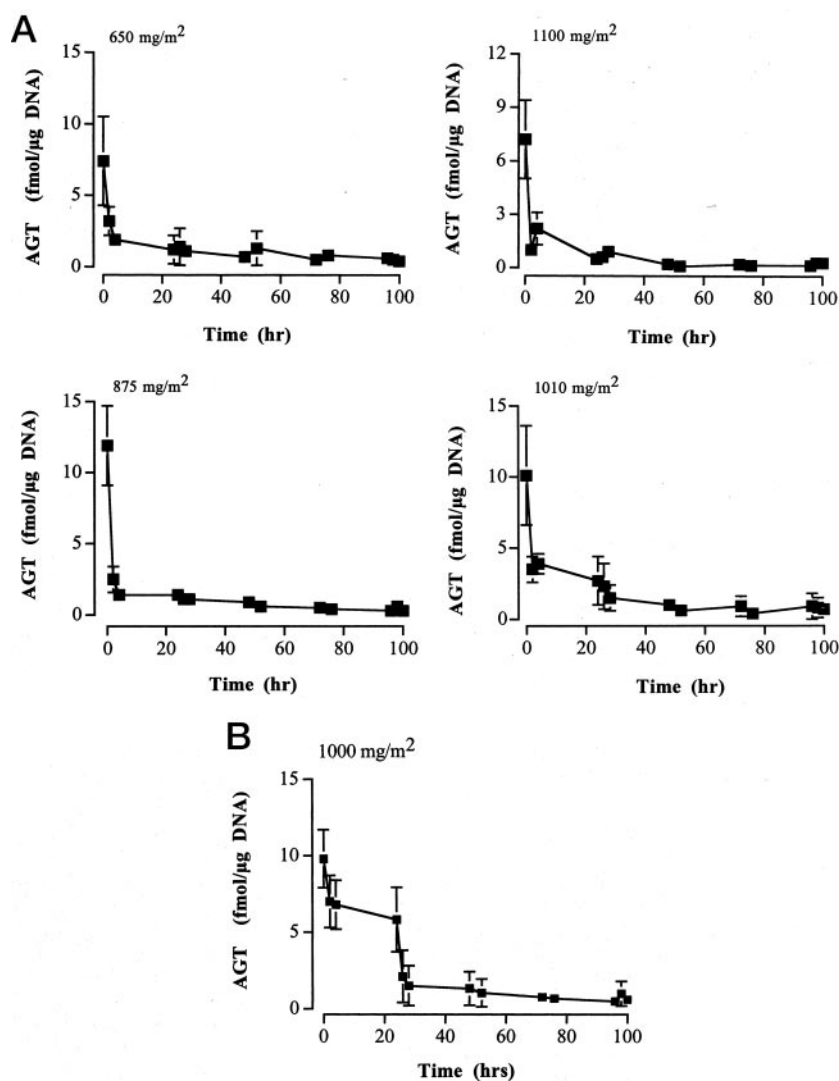


Fig. 2 A, sequential levels of AGT in PBMCs measured at baseline and during TMZ administration until 4 h after the ninth dose of TMZ. Values shown are for each cohort of patients treated with cumulative doses of 650, 875, 1010, and 1100 mg/m² TMZ administered twice a day for 5 days. B, sequential levels of AGT in PBMCs measured at baseline and during TMZ administration until 4 h after the ninth dose of TMZ. Values shown are for patients treated with TMZ once a day for 5 days at a cumulative dose of 1000 mg/m².

Depletion of AGT Activity in Tumor Tissue. This is the first clinical trial to evaluate AGT depletion by TMZ in visceral tumors. Cutting needle biopsies were obtained from hepatic metastatic deposits in 14 patients (2 at 1100 mg/m², 5 at 1010 mg/m², and 7 at 1000 mg/m²) using local anesthesia only and done under CT scan guidance (Table 1). Patient 24 had a percutaneous biopsy of cervical lymph nodes. Biopsies were performed before therapy and 2 h after TMZ treatment on day 5 of therapy. No sequelae were observed from the biopsy procedures. Evaluable paired biopsies containing tumors were obtained in 15 of 16 patients. Biopsies were not performed in the first 6 patients in the dose-escalation phase of the study (patients 1–6). A representative biopsy pair at the 1010-µg/m² dose is shown in Fig. 3.

Table 2 summarizes the biopsy data. Mean AGT activity (duplicate assays) from each biopsy segment is shown for pre-treatment and posttreatment biopsies. Baseline AGT activity showed marked variation between patients and from segment to segment in the same patient. Posttreatment, depletion of AGT

activity was variable but incomplete. In the 2 patients treated at 1100 mg/m², 1 patient showed 99.5% inactivation (undetectable levels in two of three posttreatment biopsy segments), whereas the second patient showed no decrease in activity at all. There was no relationship between the dose of TMZ and AGT depletion.

In the 1010 mg/m² group, the percentage of inactivation was between 20 and 78%. In the 1000 mg/m² group (single daily dose), the percentage of depletion of AGT activity ranged from 0 (2 patients) to 84%. No patients had total depletion of AGT activity to undetectable levels. Overall, 5 of 15 evaluable patients had <50% depletion of AGT activity in their tumor, and 10 patients had 51–99% depletion. There was no correlation between depletion and baseline AGT activity, tumor type, and dosage group. There was also no correlation between tumor response and AGT depletion in this limited study group.

TMZ Pharmacokinetics. On day 1, all subjects received TMZ 200 mg/m². The absorption of TMZ after p.o. administration was rapid. On day 1, the T_{max} occurred at 1.06, 0.83, and 1 h for the 650, 875, and 1100 mg/m² groups, respectively.

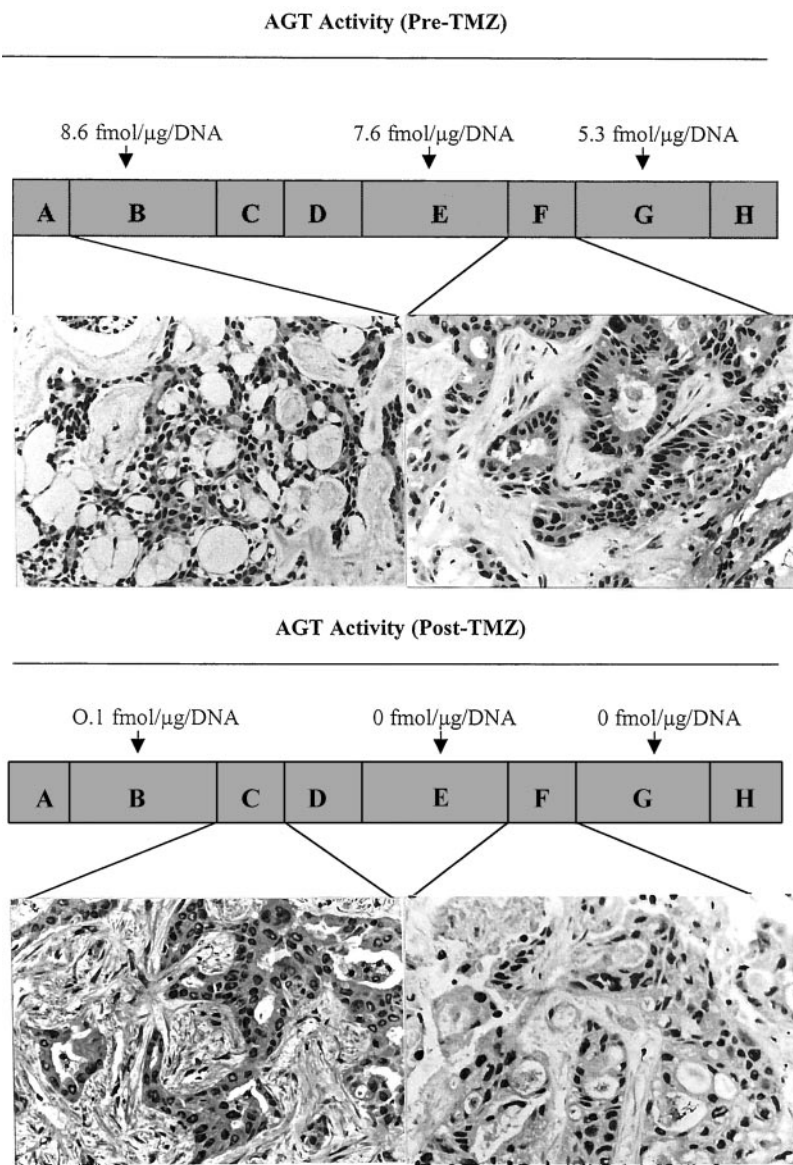


Fig. 3 Shows a representative example of pre- and posttreatment biopsies from a patient with colon cancer. The *bar* in the *top panel* of the figure represents the pretreatment needle biopsy core of tissue cut into segments labeled A–H. Segments A, C, and F were used for histological assessment (only segments A and F are shown) to ensure that tumor tissue is present and that intervening segments assessed for biochemical AGT activity truly represent tumor values. In this patient, segments B, E, and G were analyzed for biochemical AGT activity. Mean pretreatment AGT activity was 8.6, 7.6, and 5.3 fmol/ μ g DNA (duplicate assays) for segments B, E, and G, respectively. Segments D and H were used for immunohistochemical evaluation of AGT (data not shown). Similarly, the *bottom panel* shows segments A–H for the posttreatment biopsy. Biochemical AGT activity is not detectable in segments E and G and is at the limit of detection for segment B (0.1 fmol/ μ g DNA). The histological segments A, C, and F (histology for segments C and F are shown) confirm the presence of tumor tissue flanking the segments in which AGT biochemical assays were performed.

There was little intersubject variation. As expected, there was also little variation in AUC, C_{\max} , and $t_{1/2}$ on day 1 (Table 3). Pharmacokinetics on days 2 (data not shown) and 5 (Table 4) were dose proportional. The $t_{1/2}$ was unchanged (1.80, 1.96, and 1.85 for the 50, 75, and 100 mg/m² bid groups, respectively). C_{\min} values (data not shown) showed no evidence of drug accumulation from days 1–5 in any of the dosage groups. The narrow range of TMZ pharmacokinetics at each dose does not provide an explanation for the large range in values of tumor AGT depletion but does correlate with PBMC AGT depletion.

Clinical Response. We monitored clinical responses in all of our patients. Two partial responses were observed, and an additional 5 patients had prolonged stable disease for 4–6 months and an equivalent number of cycles of treatment. Only 1 of the 2 patients with PRs had a tumor biopsy (patient 19), and the tumor of this individual had a 68% AGT depletion. No cumulative hepatotoxicity or other toxicity was observed.

DISCUSSION

TMZ is a p.o. active chemotherapeutic agent with a spectrum of activity similar to that of DTIC. Unlike DTIC, it does not require hepatic activation, has good central nervous system penetration, and has predictable and reliable kinetics (14, 15). The drug has demonstrated schedule dependency in preclinical models, suggesting that the regimen of administration may be critical to its antitumor effectiveness clinically. Early pharmacodynamic-based clinical trials showed that AGT activity in PBMC used a schedule-dependent decrease with a 150 mg/m²/day dose >5 days (23). A clinical trial using a 24-h continuous infusion of TMZ effectively depleted AGT activity in PBMCs and tumor tissue (24). To date, the clinical profile of TMZ seems to mirror that of DTIC, with modest response rates in metastatic melanoma of 21% (17) but with a rapid development of resistance (median response duration of 6 months).

Table 2 AGT activity in tumor biopsies of patients after treatment with TMZ

Patient no.	TMZ dose (mg/m ²)	AGT activity ^a (fmol/μg DNA) pretreatment	No. of segments	AGT activity (fmol/μg DNA) posttreatment	No. of segments	% depletion ^b
7	1100	8.6, 7.6, 5.3	3	0.1, 0, 0	3	99.5
8	1100	7.1, 11.6, 14.1	3	10.1, 13.3	2	0
9	1010	17.3, 24.6	2	11.8, 21.6	2	20.1
10	1010	51.2, 63.2	2	9.4, 15.7	2	78.0
11	1010	23.0, 84.0	2	11.6, 24.4, 8.2	3	72.5
12	1010	25.4, 12.6	2	4.6, 6.2, 10.1, 14.7	4	53.2
14	1010	4.2, 5.8, 7.0	3	1.6, 4.8	2	43.6
20	1000	69.1, 68.8	3	1.8, 5.3, 25.8	3	84.1
21	1000	6.5, 21.8	2	31.4, 28.4	2	0
22	1000	7.3, 4.3	2	1.2, 3.6	2	58.6
23	1000	1.1, 4.0	2	2.8, 2.3	2	0
24	1000	3.0	1	0, 1.9	2	68.3
25	1000	7.2, 9.4	2	1.2, 3.5	2	71.2
26	1000	11.6, 11.0, 15.0	3	5.0, 7.1, 4.2, 7.9	4	51.6
27	1000	66.4, 77.5	2	15.6, 13.1	2	79.7

^a AGT values indicate mean AGT activity in each sequential tumor segment of a tumor biopsy. Two to four segments were analyzed per tumor biopsy depending on size and consistency of the biopsy.

^b Depletion is based on mean AGT activity (pre- and posttreatment) in each tumor biopsy.

Table 3 TMZ pharmacokinetics (day 1)

Treatment group ^a	<i>t</i> _{1/2} (h)	AUC	<i>C</i> _{max} (μg/ml)	<i>T</i> _{max} (h)
650 mg/m ² (<i>n</i> = 3)	1.67	30.59	12.06	1.06
(SD)	(0.19)	(6.56)	(3.98)	(0.87)
875 mg/m ² (<i>n</i> = 3)	1.63	33.47	12.49	0.83
(SD)	(0.23)	(3.41)	(2.72)	(0.29)
1100 mg/m ² (<i>n</i> = 2)	1.82	33.61	14.65	1.00
(SD)	(0.02)	(0.37)	(1.20)	(0.00)

^a Patients in all three groups received TMZ 200 mg/m² on day 1.

Table 4 TMZ pharmacokinetics (day 5)

Treatment group	<i>t</i> _{1/2} (h)	AUC (0–infinity)	<i>C</i> _{max} (μg/ml)	<i>T</i> _{max} (h)
50 mg/m ² BID (<i>n</i> = 3)	1.80	8.02	3.07	0.67
(SD)	(0.08)	(1.67)	(0.37)	(0.29)
75 mg/m ² BID (<i>n</i> = 3)	1.96	14.84	5.37	0.67
(SD)	(0.24)	(1.60)	(1.20)	(0.29)
100 mg/m ² BID (<i>n</i> = 1)	1.85	16.17	6.51	1.25
(SD)	(0.00)	(0.00)	(0.00)	(0.00)

As with DTIC, AGT appears to mediate a major resistance pathway for TMZ. This has led to attempts at modulation of AGT activity to overcome resistance to TMZ and related alkylating agents.

In a previous study, we used the examination of biochemical AGT activity in tumor tissue as a dose escalation end point for a Phase I trial of BG, an AGT-depleting agent. In that trial, we showed that tumor AGT activity correlates poorly with that in PBMCs, making the latter an unreliable surrogate marker (20). We have also shown that degradation rates of inactivated AGT and resynthesis rates of new AGT molecules are different in tumor tissue and PBMCs (19). Other studies have shown that pretreatment levels of AGT in tumor tissue predict poorly for response to TMZ (25). Furthermore, the importance of tumor AGT in predicting tumor response to BCNU was recently shown in a study of AGT gene promoter methylation, which results in loss of MGMT expression. In this study, patients with loss of AGT activity measured by hypermethylation of the MGMT promoter had better disease-free and overall survival after BCNU and cisplatin (26). On this basis, AGT depletion by TMZ was viewed as an important end point of drug development.

Accordingly, we designed this study to incorporate AGT activity measurements in PBMCs and tumor tissue biopsies both pre- and posttreatment in patients treated with TMZ. We show that AGT activity in PBMCs is more rapidly depleted by TMZ than in tumor tissues. PBMC AGT depletion occurs more rap-

idly (within the first 24 h) using the bid dosing schedule, and by day 5 of therapy, >95% depletion of pretreatment AGT activity was seen at all doses tested. Although AGT depletion occurred more quickly with bid than daily dosing, at the end of 5 days, AGT depletion was similar, and the MTD was the same, suggesting an equivalent biological effect. In contrast, tumor tissue AGT activity showed variable depletion that was independent of pretreatment AGT levels. Whereas all tumors had evidence of AGT activity at baseline, only 10 of 15 patients with evaluable tumor biopsies had >50% depletion of AGT in tumor samples. In 3 patients, no change was seen between pre- and posttreatment biopsies. On day 5, significant AGT activity was seen in most patients. Of note, there was no correlation between the AGT activity in PBMCs and tumor tissue. This is in sharp contrast to BG, which, at doses of 120 mg/kg, completely inactivates tumor AGT, suggesting that the combination might yield far more efficacy than TMZ alone.

It is important to note that, unlike BG, consistent levels of depletion of visceral tumor AGT by TMZ could not be achieved in tumors without toxicity. Thus, TMZ is not an optimal AGT-depleting agent, and its efficacy might be reduced as a result (conversely, it might be enhanced by AGT depletion with, *e.g.*, BG). In our study, the MTD was 1010 mg/m² but did not yield a high response rate, although 7 patients had partial responses (2 patients) or stable disease (5 patients) and received between four and six cycles of therapy. This finding is in keeping with

previous observations (14). Most of our patients were heavily pretreated. A small dose increment from 1000–1100 mg/m² resulted in hematopoietic DLT.

We also performed detailed pharmacokinetic studies in an attempt to correlate AGT activity with ambient plasma concentrations of TMZ. The $t_{1/2}$ of TMZ is short (~1 h), and by 6 h postdosing, most of the drug had disappeared from the plasma. Plasma concentrations of TMZ did not correlate with AGT activity in PBMCs, as AGT was still depleted 12 h after the last dose (on day 5) of drug. The tumor tissue biopsies were obtained 2 h after the ninth dose of TMZ, at a time when mean plasma concentrations of TMZ were between 2–4 µg/ml, a dose that we found did not deplete tumor AGT *in vitro*. *In vivo* preclinical studies show that the activity of TMZ is schedule-duration dependent (2). Likewise, Phase I clinical trials confirmed this impression. In single dose studies, no clinical responses were seen, whereas responses were seen using 5-day regimens every 28 days or 7-day regimens repeated every other week. These observations are consistent with the hypothesis that cumulative exposure of tumors to TMZ might deplete AGT activity more completely, giving rise to improved response rates.

The lack of correlation between PBMCs and tumors in the extent of AGT depletion is somewhat surprising. Because TMZ is chemically converted to the methylating metabolite, MTIC in the blood, perhaps the increased AGT depletion in PBMCs is attributable to its higher rate of conversion in blood than in tumors. Tumor blood flow may be low as well, limiting delivery of the reactive species. Perhaps the myelosuppression noted with TMZ is secondary to greater AGT depletion in the marrow than in tumors as well.

In summary, this study is the first to evaluate AGT depletion, an important target for TMZ, in normal cells and visceral tumors. While we evaluated a new drug schedule using biochemical and pharmacokinetic monitoring, we achieved toxicity without improving the end point of consistent depletion of tumor AGT. We would predict, on the basis of preclinical models, that TMZ efficacy may be directly related to the ability to deplete AGT in tumors, which is difficult to attain clinically at current dose levels. Ultimately, combination therapies with TMZ and other agents, such as BG (27), that deplete AGT might improve the therapeutic efficacy of this well-tolerated methylating agent.

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