

Original article

Sequential administration of temozolomide and fotemustine: Depletion of O⁶-alkyl guanine-DNA transferase in blood lymphocytes and in tumours

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Summary

Background: The DNA repair protein O⁶-alkylguanine-DNA alkyl transferase (AT) mediates resistance to chloroethylnitrosoureas. Agents depleting AT such as DTIC and its new analogue temozolomide (TMZ) can reverse resistance to chloroethylnitrosoureas. We report the results of a dose finding study of TMZ in association with fotemustine.

Patients and methods: Twenty-four patients with metastatic melanoma or recurrent glioma were treated with escalating dose of oral or intravenous TMZ ranging from 300 to 700 mg/m², divided over two days. Fotemustine 100 mg/m² was given intravenously on day 2, 4 hours after TMZ. AT depletion was measured in peripheral blood mononuclear cells (PBMCs) and in selected cases in melanoma metastases and was compared to TMZ pharmacokinetics.

Results: The maximum tolerated dose (MTD) of TMZ was

400 mg/m² (200 mg/m²/d) when associated with fotemustine the 2nd day with myelosuppression as dose limiting toxicity. The decrease of AT level in PBMCs was progressive and reached 34% of pretreatment values on day 2. There was however wide interindividual variability. AT reduction was neither dose nor route dependent and did not appear to be related to TMZ systemic exposure (AUC). In the same patients, AT depletion in tumour did not correlate with the decrease of AT observed in PBMCs.

Conclusions: PBMCs may not be used as a surrogate of tumour for AT depletion. Further study should concentrate on the pharmacokinetic pharmacodynamic relationship in tumour to provide the basis for individually tailored therapy.

Key words: melanoma, pharmacodynamics, pharmacokinetics, temozolomide

Introduction

Chloroethylnitrosoureas are widely used to treat solid tumours including brain tumours and metastatic melanomas [1]. They act through the formation of DNA interstrand crosslinks starting with a carbonium ion attack at the O⁶-position of the guanine and 6 to 12 hours later binding to the opposite cytidine [2, 3]. There is a good correlation between the number of interstrand crosslinks and the cytotoxicity induced by nitrosoureas [4]. The number of crosslinks depends on the ability of the cell to remove the chloroethyl group from the guanine before the crosslink is established. This removal is due to the O⁶-alkylguanine DNA alkyltransferase (AT) protein [4, 5]. AT transfers the alkyl group from the DNA to an AT internal cysteine residue in an irreversible and self-inactivating reaction [6]. Restoration of activity requires new protein synthesis [7]. AT level in human tumour cells correlates inversely with cytotoxicity of the chloroethylnitrosoureas [8–11]. *In vitro* resistance to nitrosourea can be overcome by the use of methylating agents [12] or O⁶-benzylguanine [13] to deplete AT levels in tumour cells. In the rat, dacarbazine (DTIC) has been shown to be able to deplete AT [14].

In the clinic, a sequential administration of DTIC and nitrosourea produced a 20% response rate in metastatic melanoma [15]. Lee et al. have shown that AT depletion of peripheral blood mononuclear cells was dependent on the dose of DTIC. However, the inactivation of AT reached a plateau at the highest dose level of DTIC, suggesting a saturation of its hepatic metabolism into 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC), its active metabolite [16].

Temozolomide (TMZ) is a recently synthesised imidazotetrazinone derivative. Its activation is independent of the hepatic microsomal system. At physiological pH, TMZ undergoes spontaneous degradation to generate the methylating compound MTIC [17]. TMZ is able to reverse the resistance to nitrosourea of a L1210 cell subline *in vitro* [18]. In phase I studies, leucopenia and thrombocytopenia were the dose limiting toxicities at 1000 mg/m² [19]. Antitumour activity was observed in the treatment of malignant glioma and metastatic melanoma [19–21].

Interaction between TMZ and nitrosoureas has never been studied in the clinic. We initiated a study of a sequential administration of TMZ and fotemustine (diethyl-1-3-(2-chloroethyl)-3-nitrosoureido ethyl phos-

Table 1. Patient characteristics.

Characteristics	n (range)
Total	24
Sex	
Male	14
Female	10
Mean performance status	1 (0-2)
Mean age (in years)	51 (31-71)
Primary	
Malignant melanoma	22
High-grade glioma	2
Previous treatment	
None	12
Chemotherapy	
Without DTIC	2
With DTIC	4
Immunotherapy	4
Radiotherapy	2
Route and dose of temozolomide (mg/m ² , divided over two days)	
300 i.v.	4
500 i.v.	4
700 i.v.	1
300 p.o.	3
500 p.o.	6
400 i.v./p.o.	4
400 p.o./i.v.	2

phonate), a third generation nitrosourea which showed high activity against metastatic melanoma and brain metastases from melanoma [22, 23]. The aims of the study were the following: a) to define the maximal tolerated dose (MTD) of TMZ administered four hours before a fixed dose of fotemustine b) to study the AT depletion in peripheral blood mononuclear cells and in selected tumour biopsies c) to measure the pharmacokinetics of TMZ in order to relate them to AT depletion.

Patients and methods

Patients with histologically proven metastatic disseminated melanoma or recurrent high-grade glioma were eligible for the study. The inclusion criteria were ≥ 18 years, performance status ≤ 2 according to Eastern Cooperative Oncology Group (ECOG), normal hematological, hepatic, renal and pulmonary function tests. Each patient had to give a written informed consent prior to enrolment in the study. Patients having received chemotherapy within eight weeks or having a history of lung irradiation were not included. The study was approved by the local ethics committee.

Drug formulation and treatment schedule

TMZ was supplied by the Cancer Research Campaign (CRC, London, UK) in 5 ml vials containing 150 mg TMZ in dimethylsulfoxide (DMSO). The total dose was diluted in 500 ml normal saline and was administered as a one-hour constant rate infusion. In order to achieve a substantial decrease of AT at the time of fotemustine administration, TMZ treatment was divided over two days. TMZ was planned to be administered by intravenous infusion, but, during the course of the study, TMZ became available only as a bulk powder. It was then formulated at the Pharmacy of the Centre Hospitalier Universitaire

Vaudois (Lausanne) in 20 mg and 100 mg gelatine capsules, according to the recommendations of the CRC. Capsules were ingested during a light meal. The total dose was also divided over two days. Ondansetron (8 mg i.v.) was given prophylactically as antiemetic to all patients.

Dose escalation of TMZ was performed in two groups of patients: one receiving intravenous infusion, the other oral administration. The starting dose level of TMZ was 300 mg/m² (150 mg/m² \times 2 days). Depending on the encountered toxicity, dose escalation was planned to 500 mg/m² (250 mg/m²/d) and then to 700 mg/m² (350 mg/m²/d). Toxicity was defined as dose limiting if any grade III or IV toxicity according to WHO criteria was observed [24]. Three consecutive patients were treated at each dose level. If one patient experienced a dose limiting toxicity (DLT), three additional patients were enrolled at the same dose level. If two patients reached DLT, this dose level was considered as the maximal tolerated dose (MTD). After two patients experienced DLT at the 500 mg/m² dose level, the dose of TMZ was then decreased to 400 mg/m². In order to study its bioavailability, it was administered to four patients intravenously on day 1 and orally on day 2, and to two patients orally on day 1 and intravenously on day 2.

Fotemustine was supplied by IRIS, Courbevoie, France as 4 ml vials containing 200 mg freeze dried powder that was dissolved in ethanol. The solution was diluted in a further 250 ml isotonic glucose and administered as a one hour intravenous infusion. Flask and tubing were protected from light. Fotemustine was administered at the dose of 100 mg/m² intravenously on day 2, four hours after the beginning of TMZ administration, time interval after which the maximum AT depletion by DTIC has been reported [25].

Due to the limited amount of TMZ available at the time of the study, TMZ was replaced by DTIC 500 mg/m² i.v. for the second and subsequent cycles. The treatment was repeated at a four-week interval until disease progression.

Toxicity and response evaluation

Follow-up consisted of a weekly blood cell count. Liver and lung function tests were done before the second cycle of treatment, then every two cycles. Toxicity was graded according to WHO [24]. Anti-tumour response was evaluated before the second cycle of treatment, then every two cycles according to WHO response criteria.

Pharmacokinetics

Pharmacokinetics of TMZ were studied in plasma on day 1 and 2. Two ml blood samples were collected into heparinised tubes prior to TMZ administration and at 30', 1-hour, 1-hour 30', 2-, 3-, 4-, 6-, 12- and 24-hour post administration on days 1 and 2. Blood samples were immediately centrifuged at 2000 rpm for 10' at 4 °C. An aliquot of 1 ml plasma was acidified with 0.1 ml 1 M HCl and stored at -20 °C for later analysis.

TMZ assay was performed as previously described [26, 27]. In brief, matrix components were first eliminated by solid phase extraction on a 100 mg C₁₈ cartridge, with subsequent desorption of TMZ with methanol. The resulting eluate was evaporated under nitrogen at room temperature and reconstituted in 200 μ l of 0.5% acetic acid before HPLC analysis on an ODS-column with MeOH 0.5% AcOH, 10 : 90 as solvent system. The lower limit of quantitation was 0.2 μ g/ml. The specifications of the analytical methods were in accordance with the recommendations promulgated by the Conference Report on bio-analytical validation [28]. Pharmacokinetic analysis was carried out using the computer program SIPHAR (4.0, SIMED, Créteil, France). Pharmacokinetic parameters were calculated by non compartmental approach. The elimination rate constant was determined as the slope of monoexponential curve-fit of logarithmic plasma concentrations *versus* time. The area under plasma concentration-time curve (AUC) was obtained by the linear trapezoidal method and the residual area from the last data point to infinity was estimated as the plasma concentration at this time divided by the elimination rate constant.

O⁶-Alkylguanine DNA alkyltransferase measurement

Heparinized blood samples of 10 ml were collected before TMZ and 2, 4, 8 and 24 hours after TMZ administration in prechilled syringes. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood using Ficoll-Hypaque, washed with phosphate buffered saline and stored at -80 °C. In selected patients with skin metastases of malignant melanoma, biopsies were taken one to three days before and four hours after the beginning of the administration of TMZ, time interval after which AT inactivation by DTIC reaches its nadir [25]. Biopsy samples were immediately frozen in liquid nitrogen.

AT assay of PBMCs and of tissue samples was performed as reported elsewhere [25]. Samples (1–3 × 10⁶ PBMCs or 30–90 mg tumor tissue) were sonicated, the supernatants were assayed using 10 µg (³H)-methyl DNA that had been reacted with N-(³H)-methyl-N-nitrosourea 20 Ci/mmol. Activity was expressed as fmol methyl transferred to protein per mg of protein. AT depletion was expressed as absolute decline (in fmol/mg) and as percentage of pretreatment value. The limit of quantitation of the assay was 10 fmol/mg of protein. The coefficient of variation of the same sample determined in the same day or in different days of the same week, was within 10% for PBMCs and 15% for tumour samples.

Quantitative immunofluorescence microscopy

Quantitative immunofluorescence microscopy was performed as described [29, 30]. Briefly, two paraffin slides from each tumour sample were stained in parallel, one with a monoclonal antibody against human alkyltransferase and another without antibody as a negative control. A positive control of HT29 tumour cells was also included. The slides were double-labeled with DAPI to delineate the nuclei and secondary anti-mouse FITC-labeled antibody to visualize AT-specific staining. The level of fluorescence in nuclei was calculated from the digitized epifluorescence image, and converted to molecules per nucleus using bead standards that related fluorescence intensity to molecules per unit area.

Statistical analysis

The relationship between parameters was evaluated by determination of the standard linear correlation coefficient (Pearson). The paired *t*-test was used to test whether differences between PBMCs and tumour AT pretreatment values and those at two time intervals were significantly different from zero [31, 32].

Results

Patients characteristics

Characteristics of the 24 patients are presented in Table 1. Six patients with metastatic malignant melanoma had received prior chemotherapy and four of them a regimen containing DTIC. Two patients with glioma had a recurrent disease after surgery and radiotherapy. Nine patients received TMZ as an i.v. infusion, nine patients as p.o. capsules and six patients p.o. and i.v., or the reverse sequence. Among the 24 patients, one was not assessable for toxicity due to rapid disease progression.

Dose escalation

Dose escalation in relation to toxicity is presented in Table 2. Myelosuppression was the dose limiting toxicity

Table 2. Hematological toxicity.

TMZ ^a dose (mg/m ²)		WHO toxicity grade (number of patients)				
		0	1	2	3	4
300 i.v. (n = 4)	Hemoglobin	4	–	–	–	–
	WBC	4	–	–	–	–
	Platelet	4	–	–	–	–
500 i.v. (n = 4)	Hemoglobin	3	1	–	–	–
	WBC	1	2	1	–	–
	Platelet	1	1	1	1	–
700 i.v. (n = 1)	Hemoglobin	–	–	1	–	–
	WBC	–	–	1	–	–
	Platelet	–	–	–	1	–
300 p.o. (n = 3)	Hemoglobin	3	–	–	–	–
	WBC	3	–	–	–	–
	Platelet	2	1	–	–	–
500 p.o. (n = 5)	Hemoglobin	3	1	–	1	–
	WBC	3	–	–	–	2
	Platelet	1	2	–	1	1
400 i.v./p.o. (n = 6)	Hemoglobin	3	1	–	–	2
	WBC	4	–	–	–	2
	Platelet	2	1	1	–	2

^a TMZ – indicated temozolomide.

of the sequential administration of TMZ and fotemustine. At the 500 mg/m² dose level (250 mg/m²/d) two among the five evaluable in the p.o. group had leucocytopenia and thrombocytopenia grade 3–4. In the i.v. infusion group, one among four evaluable patients had thrombocytopenia grade 3. All the patients who experienced DLT at this dose level were non pretreated. The patient at 700 mg/m² i.v. (350 mg/m²/d) also developed grade 3 thrombocytopenia. Among the six patients who received 400 mg/m² (200mg/m²/d) orally and intravenously, two reached a DLT consisting in WHO grade 4 leucopenia, thrombopenia and anemia. One of these two patients had received nitrosourea as previous chemotherapy. The dose of 400 mg/m² (200 mg/m²/d) should be considered as the MTD of TMZ when associated with fotemustine 100 mg/m² on day 2, four hours after TMZ. Duration of myelosuppression in patients who experienced WHO grade 3–4 toxicity was a median of 35 days (range 26–41 days) and was similar for leucocytes and platelets. WHO grade 1 and 2 nausea occurred in 28% of the patients despite the antiemetic prophylaxis by ondansetron. No respiratory symptoms were observed after one cycle of treatment. Lung function tests did not show any modification.

We cannot present data on the cumulative toxicity of this regimen, since only one cycle of TMZ and fotemustine was administered. Among the 19 patients who received 39 further cycles of DTIC and Fotemustine, 5 experienced WHO grade 3–4 leuco- and thrombocytopenia. The toxic events occurred after three cycles in three patients without toxicity after TMZ and fotemustine but after the first cycle in two patients who had had

a grade 4 toxicity after TMZ and fotemustine. Three patients experienced transitory lung toxicity (two WHO grade 2, one grade 4), after three (one patient) and four cycles (two patients) of DTIC-Fotemustine (i.e., after a cumulative dose of DTIC of 1500 and 2000 mg/m²).

Pharmacokinetics

Pertinent pharmacokinetics parameters are reported in Table 3. A more comprehensive detailed pharmacokinetic evaluation of TMZ is reported elsewhere [28]. Four out of six patients received the MTD (400 mg/m² divided over two days) i.v. on day 1 and p.o. on day 2 while the last two were on the opposite regimen. Under controlled conditions (standardized food intake and posture) the mean bioavailability in six patients was 0.96 ± 0.1 without any noticeable influence of the administration sequence. Pharmacokinetics of TMZ was linear after i.v. administration with AUC increasing proportionally to the dose in mg/m² ($r = 0.86$ and 0.91 for day 1 and day 2, respectively). When considering all patients having received TMZ by the oral route, the observed AUC were lower and more variable indicating a marked inter-patient variability, possibly influenced by the conditions of capsule administration.

As shown in Figure 1, there was some correlation

Table 3. Pharmacokinetic parameters of temozolomide.

TMZ dose (mg/m ²)	No. of pts	AUC (mg min/l) mean ± SD		C _{max} (µg/ml) mean ± SD	
		Day 1	Day 2	Day 1	Day 2
150 i.v.	4	1187 ± 237	1153 ± 122	7.2 ± 2.4	7.1 ± 1.8
200 i.v.	4 ^a	1704 ± 149	1376 ± 24	9.1 ± 1.8	7.5 ± 0
250 i.v.	4	2238 ± 396	1888 ± 289	12.7 ± 1.3	10.1 ± 2.0
350 i.v.	1	2819	2367	14.5	11.8
150 p.o.	3	1206 ± 200	1108 ± 306	5.3 ± 2.0	4.2 ± 1.7
200 p.o.	2 ^a	1229 ± 14	1677 ± 201	7.1 ± 1.1	8.9 ± 1.5
250 p.o.	6	1383 ± 298	1447 ± 388	7.2 ± 2.3	7.7 ± 5.7

^a AT 200 mg/m², the four patients on i.v. day 1 were the same as on p.o. day 2 and the two patients on p.o. day 1 the same as on i.v. day 2. Time range of C_{max} was after i.v. 60–98 minutes on day 1, 50–72 minutes on day 2, after p.o. 40–180 minutes on day 1 and 42–240 minutes on day 2.

(Pearson correlation test) between the degree of leucocytopenia and thrombocytopenia (percent decrease count at the nadir) and TMZ AUC after i.v. administration ($r = 0.68$, $P = 0.04$ and $r = 0.72$, $P = 0.03$, respectively). After oral administration however, TMZ AUC was not a predictive index of leucocyte and thrombocyte

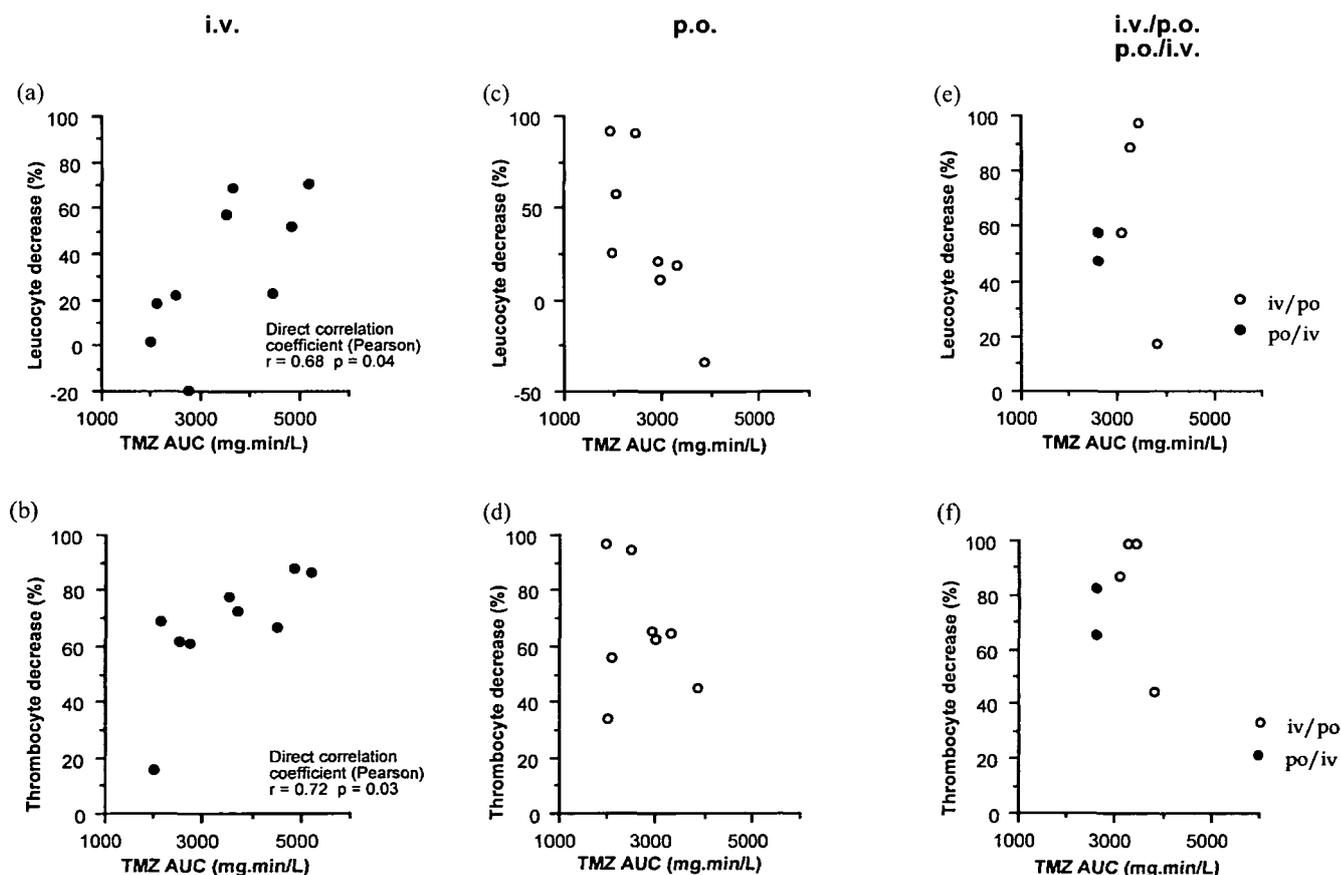


Figure 1. Relationship between temozolomide area-under-the-curve on day 1 and 2 (TMZ AUC) and the degree of leuco- and thrombocytopenia (pretreatment count minus count at the nadir/ pretreatment count) in patients who received TMZ intravenously (i.v.), orally (p.o.), intravenously on day 1 and orally on day 2 (i.v./p.o.) and orally on day 1 and intravenously on day 2 (p.o./i.v.) in association with fotemustine. TMZ was given on day 1 and 24 hours later on day 2. Fotemustine was given four hours after TMZ on day 2.

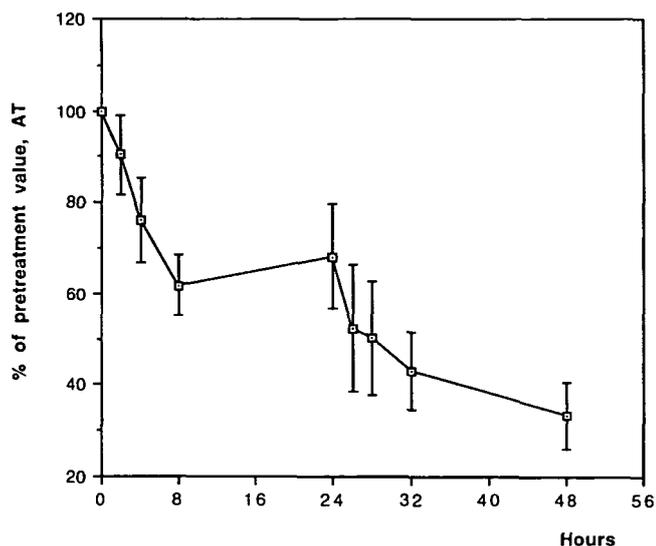


Figure 2. Mean alkyltransferase (AT) decrease in peripheral blood mononuclear cells (PBMC) of all patients \pm standard errors of the means (SEM).

toxicity. Substantial decrease in thrombocyte and in leucocyte counts was observed at lower TMZ AUC when the patients received TMZ by oral route, in comparison with those who received TMZ by i.v. infusion.

Pharmacodynamic assessment of AT depletion

AT level in PBMCs was measured in 20 patients on day 1, in 19 patients on day 2 and in 6 patients also after DTIC, on day 29. Pretreatment values were scattered ranging from 121 to 1147 fmol/mg protein (mean 376 fmol/mg). In the whole group, mean AT levels after treatment showed a progressive and cumulative decrease corresponding to (\pm SEM) 62% \pm 8% of pretreatment value after eight hours, 52% \pm 14% at the time of fote-mustine administration and reaching its lowest value at 48 hours with 34% \pm 7% (Figure 2). AT levels at eight hours on day 1 and at four hours on day 2 were statistically different from pretreatment values ($P < 0.0004$). The absolute AT decline (pretreatment minus AT level at the nadir) was directly related to the pretreatment AT level with a correlation coefficient of 0.97. The most prominent feature was, however, a wide interindividual variation in the extent of the decline of AT, even in patients treated at the same dose level. Figure 3, for example, shows the AT levels in the group of patients who were given TMZ 250 mg/m²/day by i.v. infusion. Due to this high interpatient variability, we were unable to assess differences between doses and routes of administration. Similarly AT decrease could not be related to TMZ systemic exposure (TMZ plasma AUC, Figure 4). Complete AT depletion, was observed at each dose level, in five patients. AT decrease was observed in patients who had received previous treatment as well as in non pretreated patients. However, two out of three patients who had received DTIC as previous chemotherapy had no or minimum AT depletion (nadir at 108 and 77%

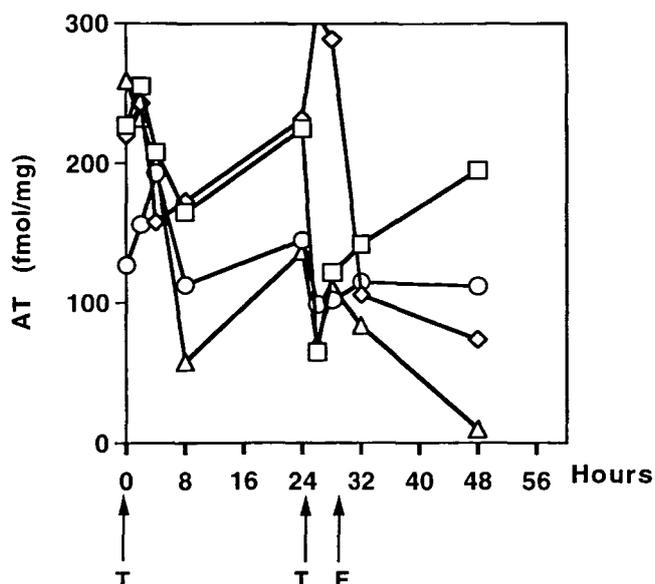


Figure 3. Alkyltransferase (AT) levels (fmol/mg) in peripheral blood mononuclear cells in four patients who received Temozolomide (T) 250 mg/m² i.v. T was given on day 1 and 24 hours later on day 2. Fote-mustine (F) was administered four hours after temozolomide on day 2.

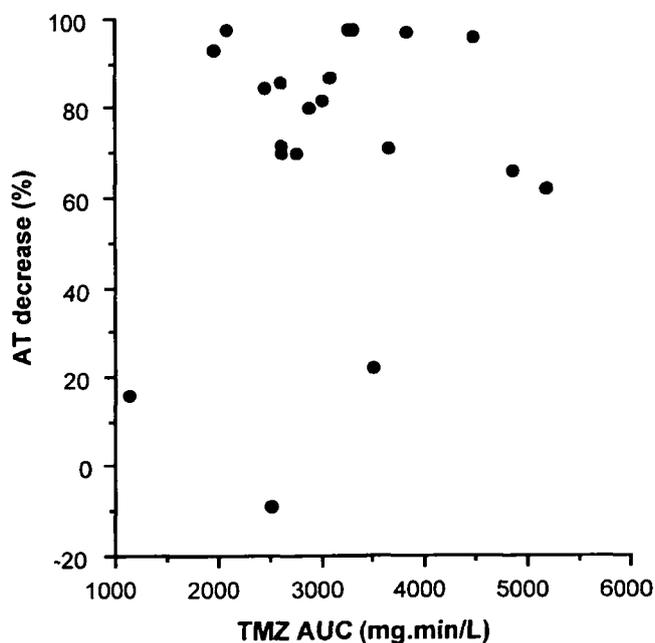


Figure 4. Relationship between the temozolomide area-under-the-curve on day 1 and 2 and the degree of alkyltransferase (AT) depletion (percent decrease at the AT nadir) in peripheral blood mononuclear cells (PBMCs).

of pretreatment level, respectively). Ten patients had partial or complete recovery of their initial AT level on day 1 and five on day 2. PBMCs AT depletion was not related to myelosuppression (data not shown).

On day 29, AT level reached the pretreatment values in five among six assessed patients. One patient retained a low AT level at 22% of its pretreatment value. AT depletion achieved by 500 mg/m² DTIC i.v. on day 29

was more rapid than that observed on day 1 and 2 after TMZ at 250 mg/m²/d i.v., but the nadir of AT depletion was similar.

Six patients had biopsies of melanoma skin metastases before and four hours after TMZ administration. There was also wide interindividual variations in pretreatment levels of AT in tumour (mean value 296 fmol/mg, range 133–488 fmol/mg). After TMZ, mean AT level was 56% ± 15% of pretreatment level in tumour. It decreased from 19% to 72% in all but one patient in whom AT level increased by 16%. As shown in Table 4, there was, in three patients, a discrepancy between AT inactivation in peripheral blood lymphocytes and in tumours. Two patients (patients no. 1 and no. 6, Table 4) had a decrease in their tumour AT activity and an increase in their PBMCs AT, one patient (patient no. 3, Table 4) had an increase in tumour AT and a decrease in PBMCs AT. The extent of AT depletion in tumor could not be related to TMZ dose in this small group of patients. The maximum AT decrease was even observed in a patient treated at the TMZ lowest dose level (patient no. 1, Table 4).

Quantitative immunohistochemistry of AT gave results similar to those obtained with the biochemical method. For example, a section from a melanoma skin metastasis biopsy taken prior to TMZ treatment showed much greater AT-specific immunofluorescence than a section taken 4 hours after treatment (Figure 5). Quantitation of the nuclear AT-specific staining in this patient showed a reduction from an average of 44,000 AT molecules per nucleus before to 16,000 molecules per nucleus after treatment. The AT appeared to be localized in the cytoplasm prior to treatment (panel a), as observed previously [30], and was depleted from both the nucleus and cytoplasm after TMZ treatment (panel b).

Clinical assessment and relationship with AT depletion

The antitumour response was not a main goal of this study. Tumour evaluation on day 28 revealed tumour progression in five patients and stable disease in 19 patients. Three patients with stable disease had minor tumour regression: one had the disappearance of multiple skin metastases with the persistence of visceral, pulmonary and hepatic lesions, another had a radiological improvement of a lung infiltrate and the third had a 25% radiological decrease of an iliac lymph node. The 19 patients with stable disease received the treatment as planned with DTIC and fotemustine. Objective responses were observed in three patients (16%): one complete response in the patient with the improvement of a lung infiltrate and 2 partial remissions (in the patient with the disappearance of skin metastases and in another one with lung metastases).

AT levels in melanoma were measured in 6 out of 24 patients. This small number did not allow to draw definite conclusion regarding the relationship between the AT decrease in the tumour and the clinical response. Noticeably however, the patient with melanoma skin metastasis experiencing a complete response had a 70%

Table 4. AT level in peripheral blood mononuclear cells and in tumour (six patients).

TMZ ^a dose	Patient number	PBMC AT level (fmol/mg) ^b		Tumour AT level (fmol/mg)	
		T ₀	T _{4h}	T ₀	T _{4h}
150 mg/m ² /d i.v.	1	104	213	133	25
200 mg/m ² /d i.v.	2	621	263	256	163
200 mg/m ² /d i.v.	3	1147	298	488	570
200 mg/m ² /d p.o.	4	718	475	458	134
200 mg/m ² /d p.o.	5	631	447	212	153
250 mg/m ² /d i.v.	6	127	156	228	80

^a TMZ – indicates temozolomide.

^b PBMC AT level for alkyl-transferase level in peripheral blood mononuclear cells.

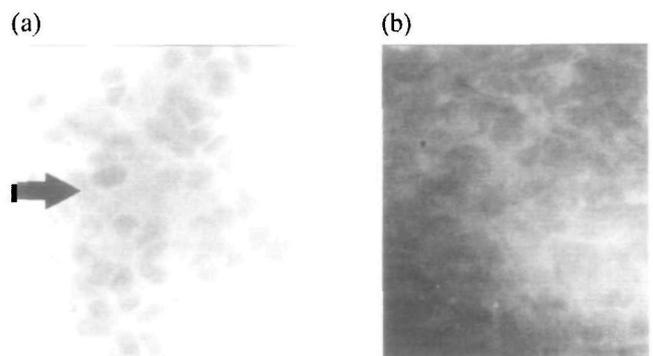


Figure 5. Alkyltransferase (AT) immunofluorescence in sections from biopsies taken before (a) and four hours after (b) Temozolomide. The AT-specific antibody staining in the sections was visualized with FITC-secondary antibodies. In the sample taken before (a) note the cytoplasmic localization with relatively low nuclear fluorescence (arrow).

decrease of AT level in the tumour biopsy (patient no. 4), while another patient (no. 3) with rapid tumour progression had a 17% increase of AT level in the excised tumour.

Discussion

Part of the resistance to nitrosourea is mediated through the O⁶-alkylguanine-DNA alkyltransferase protein (AT), which is depleted by TMZ *in vitro* and *in vivo* [33, 34]. To our knowledge, this is the first report of the clinical application of the sequential administration of TMZ and nitrosourea. TMZ administration was divided over two days to achieve a substantial decrease of AT at the time of fotemustine administration. The main toxicity was myelosuppression, including thrombocytopenia and anemia. The MTD of TMZ was 400 mg/m² divided over two days when given in combination with fotemustine 100 mg/m² the second day, four hours after TMZ.

When the study was initiated, TMZ was available as injection form only, it then became available as oral formulation. This form is currently approved for general use. Thrombocytopenia and leucocytopenia were more pronounced when TMZ was administered by the oral route as seen in Figure 1. The toxicity was however not predictable but occurred generally at lower AUC as

compared to i.v. administration. Since we confirmed that the bioavailability of TMZ was close to 1 (i.e. $AUC\ p.o./AUC\ i.v. = 1$), the difference, observed in this small group of patients, reflects interpatient variability and susceptibility to TMZ rather than route dependency. However, some unrecognized effect of TMZ administered by oral route cannot be fully excluded.

For further phase II trials of TMZ in association with a nitrosourea, a starting dose of $300\ mg/m^2$ ($150\ mg/m^2/day$) divided over two days should be recommended. This dose should be escalated in patients without toxicity.

However, the cumulative toxicity of this regimen, and in particular the risk of lung toxicity, is not known, since only one cycle of TMZ and fotemustine was administered in this study. The pulmonary toxicity that occurred in three patients after further treatment with DTIC and fotemustine has been previously reported after this regimen, and a cumulative dose of DTIC of $1500\ mg/m^2$ [15]. Signs of such a toxicity should be carefully looked for in future phase II trials of TMZ in association with a nitrosourea.

TMZ clearly showed a linear pharmacokinetics with AUC increasing proportionally with dose after i.v. administration. Pharmacokinetic parameters and bioavailability values found in this study [28] are in good accordance with those previously published [19].

The progressive and cumulative AT depletion in PBMCs with wide interindividual variation has been previously reported after DTIC [16, 25] and after TMZ [34]. It has been hypothesized that interindividual variability in AT depletion was due to differences in pharmacokinetics of TMZ. However, in our study, the variability of TMZ pharmacokinetics did not influence the absolute, relative or cumulative AT decrease. AT depletion by DTIC was dose dependent [16] but we could not demonstrate the same dose-response effect after TMZ. Indeed, there was no clear relationship between the TMZ dose administered and the extent of AT depletion.

Previous reports have mentioned the heterogeneity of AT level in liver metastases from colon cancer [32] and in skin metastases from melanoma [35]. In the same patient a variation of up to 400% among lesions was also observed [35]. Although we cannot rule out that the lower AT levels we observed in melanoma skin metastases after treatment might be due to interlesional heterogeneity, the consistency of the decrease strongly suggests that it is an effect of the treatment. Interestingly enough, AT depletion in tumour and in PBMCs were not related. An analogue observation has been reported in seven patients with liver metastases from colon cancer treated with streptozotocin and BCNU [32]. These observations indicate that depletion of AT in PBMC is a marker for production of O^6 -alkylguanine in DNA, but the extent of depletion in PBMCs may not accurately reflect the extent of AT depletion in metastases from melanoma and colon cancer. Inaccessibility prevented AT decrease assessment in patients with glioma. A direct relationship between AT depletion in glioma and in PBMCs can therefore not be excluded.

In our small group of patients maximum AT depletion was observed at all TMZ dose levels in PBMCs and at the lowest dose level in melanoma. Moreover, AT depletion in tumour and in PBMCs could not be directly related to TMZ exposure. If the aim of the TMZ treatment is to sensitize tumour to the action of chloroethylnitrosoureas, it may not be necessary to use the MTD. Modulation of AT depletion by TMZ might be better achieved by individualizing the dose of TMZ. Most of dose adjustment strategies described to date rely on pharmacokinetic measurements and on dose adjustment based on pharmacokinetic–pharmacodynamic models [36]. In clinical practice, it has been successfully applied to define the doses of methotrexate, carboplatin, etoposide, teniposide, 5-fluorouracil and suramin [37]. Modulation of AT could be a future approach and should concentrate on AT measurement in tumour. The ultimate aim is to provide a model, based on the measurement of AT and its depletion in tumour, to tailor the administration of AT depleting agents to the individual patient. Among the agents designed to inactivate AT, O^6 -benzylguanine has recently received considerable attention. Unlike TMZ, O^6 -benzylguanine acts as a direct inactivator of AT without interaction with DNA and has no intrinsic tumour activity [38, 39]. In a phase I study in patients with glioma, it depleted completely AT levels in glioma without occurrence of toxicity [39]. Phase I trials defining the optimal dose of the chloroethylnitrosourea carmustine to be combined with O^6 -benzylguanine are ongoing. Preliminary reports seem however to show a substantial enhancement of the hematologic toxicity of carmustine by O^6 -benzylguanine requiring the dose of carmustine to be decreased [40]. It is however still not clear which approach offers the best therapeutic window: either a complete depletion by agents such as O^6 -benzylguanine but resulting in increased end organ toxicities necessitating a decrease in the dosage of the alkylating agent or only a partial depletion by methylating agents such as TMZ which have intrinsic cytotoxic activities.

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