Pharmacokinetics of 3-Methyl-(triazen-1-yl)imidazole-4-carboximide following Administration of Temozolomide to Patients with Advanced Cancer

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ABSTRACT
The antitumor activity of temozolomide (TMZ) is believed to arise via formation of the reactive, alkylating metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboximide (MTIC), which is produced by chemical hydrolysis of the parent drug. MTIC has not been quantitated in plasma or urine following administration of TMZ to patients. We developed a sensitive, specific method for the determination of MTIC levels in plasma, based on reverse-phase high-pressure liquid chromatography of the supernatant that is obtained by methanol precipitation of plasma proteins. Due to poor stability under physiological conditions, determination of MTIC required rapid specimen processing, precipitation of plasma proteins with methanol, and storage of the methanolic supernatant at -70°C. The pharmacokinetics of MTIC were studied in 15 patients who received 125-250 mg/m² TMZ. Peak plasma concentrations of 0.07-0.61 μg/ml MTIC were observed approximately 1 h after a p.o. dose of TMZ. Appearance and disappearance (t1/2, 88 min) of the reactive metabolite paralleled the appearance and disappearance of TMZ in plasma. The mean values of the metabolite peak plasma concentration and AUC were 2.6% (range, 1.6-4.6%) and 2.2% (range, 0.8-3.6%), respectively, of the values for TMZ. MTIC did not accumulate in plasma following five consecutive daily doses of TMZ.

INTRODUCTION
TMZ (Fig. 1) is an imidazotetrazine with potent, broad-spectrum antitumor activity in rodent tumor models and with antiproliferative activity against cultured human tumor cell lines (1, 2). Interest in the continued development of TMZ stems from the encouraging results of European Phase I and II trials. During Phase I evaluation, clinical activity was detected in 4 of 23 patients with melanoma, in 2 patients with high-grade glioma, and in 1 patient with drug-resistant mycosis fungoides (3). These results have been confirmed in subsequent Phase II trials, in which a 21% response rate was found for patients with metastatic melanoma (4), and clinical improvements were noted for 5 of 10 patients with high-grade astrocytomas (5).

There is considerable evidence to suggest that MTIC is a key intermediate in the antitumor activity of TMZ. MTIC was identified as the product of mild alkaline hydrolysis of TMZ (1) and was detected in the culture medium from incubations of TMZ with tumor cells (2). This highly reactive imidazotriazene had antiproliferative activity against tumor cells in culture (2), presumably due to methylation of DNA. Covalent binding of methyl moieties from [3H-methyl]-MTIC to the O6 position of guanine was observed following incubation with L929 fibroblast DNA (6). Consistent with this result, several reports have demonstrated an inverse relationship between TMZ cytotoxicity and the activity of AGT, the DNA repair enzyme that selectively removes O6-methyl and other alkyl adducts with guanine residues in DNA. The sensitivities of leukemic blast cells (7) and several human tumor cell lines (8) to TMZ were inversely related to cellular levels of AGT, but TMZ and a related analogue, DTIC, depleted AGT (9, 10). Depletion of AGT by O6-benzylguanine (9, 11) and O6-methylguanine (12) increased the sensitivity of cells to TMZ.

The pharmacokinetics of MTIC have not been reported in previous preclinical and clinical investigations of TMZ, presumably due to the poor aqueous stability of MTIC, which imposes severe constraints on analytical methodology. Because MTIC is an important, active metabolite of TMZ, we examined the plasma disposition of MTIC as part of our Phase I evaluation of TMZ in adults. Because MTIC is unstable under physiological conditions, we developed a sensitive, specific HPLC assay for the analysis of MTIC in plasma, based on rapid sample preparation techniques, and we used the assay to characterize the pharmacokinetics of MTIC following administration of TMZ.

MATERIALS AND METHODS
Chemicals
MTIC and AM were provided by Schering Plough. TMZ and DTIC were supplied by NIH. HPLC-grade acetonitrile, methanol, and ammonium phosphate were obtained from commercial sources and used as received.

Solutions
Stock solutions of MTIC and DTIC were dissolved in methanol in borosilicate glass vials that were sealed with
Teflon-lined screw-on caps. Solutions were prepared fresh each day and kept on dry ice to prevent degradation.

Patients

The study population included patients with advanced cancer who received treatment with TMZ p.o. for 5 consecutive days in a Phase I trial at the Mayo Clinic (Rochester, MN). Four patients whose prior treatment included nitrosoureas received 125 mg/m² TMZ. Eleven patients whose prior treatment did not include nitrosoureas received 200–250 mg/m² TMZ. Patients were at least 18 years old and had histologically confirmed measurable or evaluable malignancy, a life expectancy of >12 weeks, and no other more conventional means of therapy that would offer reasonable hope of cure or significant palliation. Patients were also required to have good performance status (Eastern Cooperative Oncology Group performance status of ≤2), adequate oral nutrition (≥1200 calories/day), platelet counts of ≥130,000/mm³, serum creatinine levels of ≤0.3 mg/dl above institutional normal limits, and direct bilirubin levels within institutional normal limits. WBC counts for patients with and without prior nitrosoureas were ≥3500/mm³ and ≥4000/mm³, respectively.

Specimens

Blood samples (5 ml) were collected in heparinized tubes and immediately chilled in an ice-water bath. Plasma was separated by centrifugation (3000 rpm for 10 min) in a centrifuge maintained at 4°C. A 1-ml aliquot of plasma was transferred to a microcentrifuge tube containing 0.1 ml of 1.0 N HCl for TMZ analysis. A second aliquot of plasma (400 μl) was transferred to a microcentrifuge tube containing two volumes (800 μl) of chilled (−20°C) methanol. The contents of the tube were mixed vigorously and allowed to stand on ice for 5 min, and the precipitated proteins were pelleted by centrifugation at 10,000 rpm for 2 min. The supernatant was transferred to a silanized microcentrifuge tube and stored at −70°C until HPLC analysis for MTIC.

Sample Preparation and Analysis

MTIC. Standard curve samples (0.02–1.0 μg/ml) were prepared by addition of an aliquot of a standard MTIC solution to plasma (1 ml) that was acidified with 0.1 ml of 1.0 N HCl. A 400-μl aliquot was immediately transferred to a microcentrifuge tube containing 800 μl of ice-cold methanol to precipitate plasma proteins. Following centrifugation (10,000 rpm for 2 min), the aqueous methanol supernatant was transferred to a silanized microcentrifuge tube and analyzed immediately.

HPLC separation was achieved using a Zorbax Stablebond-Cyano (4.6 × 150 mm) analytical column (Mac-Mod Analytical) and a Brownlee Cyano Newguard (15 × 3.2 mm, 7 μm) precolumn (Chromtech). The mobile phase was composed of methanol:50 mM ammonium phosphate (20:80), pH 6.50, and was delivered at a flow rate of 1.0 ml/min. Effluent was monitored with a Shimadzu SPD-10AV UV-Vis detector at 318 nm. Prior to chromatography, 10 μl of 1 μM DTIC (internal standard) was combined with 50 μl of plasma supernatant and 50 μl of 50 mM ammonium phosphate, pH 6.50. The mixture was shaken vigorously, and 50 μl were injected immediately by manual injection. Samples were prepared and injected one at a time.
TMZ. The HPLC assay for TMZ in plasma was based on procedures published by Newlands et al. (3). TMZ was isolated by ethyl acetate extraction of plasma following addition of ethazolastone [8-carbamoyl-3-ethylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, the ethyl analogue of TMZ] as the internal standard. Extracts were chromatographed by reverse-phase HPLC on a Lichrosorb C18 (EM Science) analytical column and a Newguard RP18 (Brownlee) precolumn. Elution was accomplished with a mobile phase composed of methanol:50 mM phosphoric acid (10:90, v/v), pH 2.0. TMZ and ethazolastone were detected by UV absorption at 325 nm.

Data Analysis

The terminal elimination rate constant (kₘ) was calculated by linear least squares regression of the terminal exponential phase of the graph of natural logarithm of the plasma concentration versus time. AUC was determined by trapezoidal approximation from the start of treatment to the last detectable plasma concentration (Cₜₐₙₜ), with residual area (AUCₜ) after Cₜₐₙₜ calculated by AUCₜ = Cₜₐₙₜ/kₘ. The elimination half-life was calculated by tₑₙ = 0.693/kₘ. The statistical significance of differences in experimental groups was made by independent t test, with P ≤ 0.05 as the criterion for significance.

RESULTS

Our investigation of the pharmacokinetics of MTIC following administration of TMZ required the availability of the sensitive specific assay for determination of MTIC in plasma. Due to the rapid degradation of MTIC under physiological conditions and the limitations imposed by collection of specimens from human subjects in a clinical investigation, we carefully evaluated HPLC conditions and sample preparation procedures that were suitable to a molecule with very poor aqueous stability.

HPLC Assay. Efficient separation of TMZ, MTIC, and DTIC was accomplished with reverse-phase HPLC on a cyano-propyl column that was eluted with a mobile phase consisting of methanol:50 mM ammonium phosphate (20:80), pH 6.5. Precipitation of plasma proteins with methanol followed by centrifugation provided aqueous methanol supernatants that were free of interferences when UV absorbance was monitored at 318 nm (Fig. 2). Recoveries of MTIC and DTIC from plasma samples containing known amounts of MTIC and DTIC were >95% when the supernatants were chromatographed immediately following precipitation of plasma proteins with methanol.

Due to the susceptibility of MTIC to hydrolysis in aqueous solutions and to anecdotal reports of poor stability in frozen plasma, we evaluated MTIC stability in whole blood, plasma, and aqueous methanol supernatants. MTIC was rapidly hydrolyzed (tₑₙ/2, 2 min) in whole blood and plasma at 37°C (Table 1). Hydrolysis of MTIC in whole blood was delayed when whole blood samples were placed on ice (tₑₙ/2, 74 min).

MTIC decomposition in plasma was reduced by precipitation of plasma proteins with methanol; the half-life of MTIC in the aqueous methanol supernatant at room temperature was ~51 min. When the supernatants were placed on ice, ~<5% decomposition of MTIC was detected after 4 h. We also assessed recovery of MTIC following long-term storage of plasma samples and aqueous methanol plasma supernatants. Plasma concentrations of MTIC were reduced by 50% and 25% when frozen plasma samples were stored for 7 days at -20°C and -70°C, respectively. In contrast, MTIC degradation was greatly reduced if aqueous methanol plasma supernatants rather than plasma samples were stored at -20°C or -70°C. Less than 5% of added MTIC was degraded over the 7-day period (Table 1). Although plasma protein precipitation provided adequate stability for the preparation and storage of specimens, manual injection of the aqueous/organic supernatants was required in the absence of a low-temperature autoinjector.

Because MTIC undergoes rapid degradation in whole blood, we were concerned about losses of the metabolite during the early portion (e.g., plasma isolation) of the work-up procedure. The mean (± SD) recovery values (n = 5) of MTIC from whole blood containing 0.15, 0.4, and 1.0 μg/ml at 37°C were 79% (± 18%), 87% (± 20%), and 93% (± 15%), respectively. The small differences in recovery values were not significant (P > 0.20).

MTIC plasma standard curves were linear (r² > 0.99) over the range 0.05-1.0 μg/ml. The lower limit of quantitation was 0.05 μg/ml. The accuracy and precision of the method were determined with quality assurance specimens containing 0.075,
Pharmacokinetics of MTIC and TMZ. The pharmacokinetics of MTIC and TMZ were characterized in 15 patients who received p.o. doses of TMZ in a National Cancer Institute-sponsored Phase I trial at the Mayo Clinic. Four patients who were previously treated with nitrosoureas received 125 mg/m² TMZ, whereas the remaining 11 patients received 200–250 mg/m² TMZ. TMZ pharmacokinetics for these patients were similar to those found for the full cohort of 36 patients. Complete details of the Phase I trial have been published separately (13). In brief, following rapid absorption of the p.o. dose (tₚₑᵃكسبₕₖ, 1 h), TMZ plasma levels declined with an elimination half-life of 109 min. TMZ clearance was independent of dose, and no accumulation was noted following administration of five daily doses. AM, a TMZ metabolite formed by oxidation of the carboxamide moiety, was also detected in patient plasma; however, most concentrations were below the level of reliable quantitation (0.02 μg/ml).

MTIC was detected in patient plasma following p.o. administration of 125–250 mg/m² TMZ. Although plasma MTIC concentrations were low compared to TMZ, measurable concentrations of the metabolite were observed for up to 6 h following an p.o. dose of TMZ. The appearance and disappearance of MTIC in plasma paralleled the absorption and elimination of TMZ (Fig. 3). Peak plasma concentrations of 0.07–0.61 μg/ml MTIC were observed approximately 1 h (range, 20–150 min) following a p.o. dose of TMZ and were 2.6% (range, 1.6–4.6%) of the peak plasma concentrations of TMZ. The mean peak MTIC plasma concentrations for patients who were treated with 200–250 mg/m² TMZ were 60% greater than the mean value for patients treated with 125 mg/m² TMZ (Fig. 4). Plasma elimination of MTIC followed apparent first-order kinetics, with a half-life of 88 min.

MTIC AUC values were 2.2% (range, 0.8–3.6%) of those observed for TMZ (Table 2), and the proportion did not change with TMZ dose. MTIC AUC values calculated for 200–250

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**Table 1** Summary of MTIC stability

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Plasma</th>
<th>Whole blood</th>
<th>Methanol supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C (Room)</td>
<td>2.1 min</td>
<td>2.1 min</td>
<td>51 min (tₑ₉₅ = 3.8 min)</td>
</tr>
<tr>
<td>4°C</td>
<td></td>
<td></td>
<td>69 h (tₑ₉₅ = 5.1 h)</td>
</tr>
<tr>
<td>−20°C</td>
<td></td>
<td>74 min (tₑ₉₅ = 5.5 min)</td>
<td>Stable (tₑ₉₅ = 7 days)</td>
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<tr>
<td>−70°C</td>
<td></td>
<td></td>
<td>Stable (tₑ₉₅ &gt; 9 days)</td>
</tr>
</tbody>
</table>

*₉₅, length of time in which the concentration will fall to 95% of the initial value.
mg/m² TMZ were approximately 2-fold greater than the values calculated for 125 mg/m² TMZ. When TMZ was administered for 5 consecutive days, there was no difference between day 1 and day 5 AUC values.

**DISCUSSION**

We have developed a new, sensitive, specific HPLC assay for the alkylating metabolite MTIC and have used that assay to investigate the pharmacokinetics of MTIC following administration of TMZ, an investigational agent with documented activity in melanoma and brain tumors. Our data are consistent with a role for MTIC in the antitumor activity of TMZ.

The rapid degradation of both TMZ and MTIC under physiological conditions posed practical problems for the quantitative determination of MTIC in patient plasma. Initial degradation of MTIC in whole blood was delayed when specimens were chilled to 4°C. Artificial formation of MTIC by hydrolysis of TMZ was prevented by the acidification of plasma. Further degradation of MTIC in plasma was minimized when plasma samples were precipitated with methanol and the aqueous methanol supernatant was stored at -70°C. Finally, the aqueous organic supernatants were injected manually to avoid degradation of HPLC samples in autosampler tubes at room temperature. Thus, losses of MTIC were <15% over the full work-up period. The low limit of sensitivity (0.05 μg/ml) permitted determination of MTIC plasma concentrations following p.o. administration of TMZ. The assay variability was greater than 10%, but this was not unexpected, given the poor stability of MTIC.

With the appropriate analytical methodology, we detected MTIC in patient plasma following administration of TMZ and found that plasma exposure of MTIC was much less than that of parent drug. Thus, MTIC is formed directly from TMZ, and MTIC degradation is rapid \( t_{1/2} = 2 \) min and extensive. There is little recovery of TMZ in urine, and recovery of AM, the only other known route of TMZ metabolism, is minimal (13, 14).

Although plasma concentrations of MTIC are low, we presume this degradation pathway represents the principle route of TMZ elimination and is responsible for TMZ antitumor activity *in vivo*. The mechanism of action of TMZ is unknown, but it is believed to be associated with methylation of \( O^\beta \)-guanine moieties in guanine-rich DNA sequences by the methyl cation that is produced by decomposition of MTIC (Fig. 1; Ref. 15). MTIC has antiproliferative activity that is attributed to \( O^\beta \)-guanine methylation of DNA (6). The sensitivity of cultured tumor cells to TMZ is associated with levels of AGT, the enzyme that repairs \( O^\beta \)-guanine adducts (8).

DTIC, an imidazotriazene that is related to TMZ, is also a precursor of MTIC (2). In contrast to TMZ, the formation of MTIC from DTIC is dependent upon cytochrome P450-catalyzed \( N \)-demethylation of the dimethyltriazeno substituent of DTIC (2, 6). Despite the encouraging antitumor activity of DTIC that is observed in mice, limited antitumor activity has been observed in patients. One explanation for the disappointing clinical results with DTIC is the limited and variable metabolic activation of parent drug to MTIC. In contrast to TMZ, which does not require the presence of active mouse liver microsomes to exhibit cytotoxicity against cultured tumor cells, the cytotoxicity of DTIC against cultured tumor cells is dependent upon the presence of active mouse liver microsomes (2). The availability of a sensitive, specific assay for MTIC now permits investigation of the pharmacokinetics of this active metabolite in patients treated with DTIC in a clinical trial that is now underway at the Mayo Clinic.

In conclusion, development of a new assay for plasma MTIC permitted characterization of MTIC pharmacokinetics following administration of TMZ. TMZ appears to be an ideal produg of MTIC because it may be administered p.o., with essentially complete bioavailability, and it yields MTIC as the primary product. Our assay will be equally important in characterizing the role of MTIC in the pharmacokinetics and anti-

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**Table 2** Patient systemic exposure (AUC) to TMZ and MTIC following administration of TMZ on days 1 and 5 of a 5-day administration schedule

<table>
<thead>
<tr>
<th>Dose (mg/m²)</th>
<th>Day 1</th>
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<th>Day 5</th>
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<tr>
<td></td>
<td>TMZ (μg/ml/min)</td>
<td>MTIC</td>
<td>% TMZ</td>
<td>TMZ (μg/ml/min)</td>
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<tr>
<td>125</td>
<td>1264</td>
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<td>200*</td>
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<td>1729</td>
<td>38.4</td>
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<td>2120</td>
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<td>41.0</td>
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<td>2022</td>
<td>31.8</td>
<td>1.8</td>
<td>2112</td>
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*No prior nitrosoureas.
tumor activity of DTIC in a clinical trial that is now underway at the Mayo Clinic.

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