Preclinical Pharmacokinetic and Pharmacodynamic Evaluation of Metronomic and Conventional Temozolomide Dosing Regimens

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ABSTRACT
Metronomic dosed (MD) chemotherapy as opposed to conventional dosed (CD) chemotherapy is considered an alternate strategy to target angiogenesis and limit host toxicity. Although this approach is promising, there has been no attempt to define optimal metronomic dosing regimens by integrating pharmacokinetic (PK) with pharmacodynamic (PD) measurements. The aim of this study was to compare the pharmacokinetics and pharmacodynamics of temozolomide [TMZ, 8-carbamoyl-3-methylidazo(5,1-d)-1,2,3,5-terrazin-4(3H)-one] after MD and CD regimens. In vivo studies were carried out in xenografted athymic rats treated with either 18 mg/kg/day TMZ for 5 days or 3.23 mg/kg/day TMZ for 28 days. PK studies were performed on the first and last days of dosing. PD measurements consisted of gene and protein expression of various angiogenic markers, tumor size, tumor pH, and interstitial fluid pressure (IFP). The results demonstrated that the PK parameters (total clearance, volume of distribution, and tumor/plasma accumulation) were quite similar for MD and CD groups, consistent with the linear PK properties of TMZ. Both TMZ treatment schedules caused a significant decrease in IFP and tumor size compared with vehicle control treatment, demonstrating comparable effectiveness of MD and CD regimens. Using real-time polymerase chain reaction and Western blot analyses, some differences were noted in expression levels of vascular endothelial growth factor and hypoxia inducible factor-1α, suggesting that the MD regimen may be superior to the CD regimen by preventing tumors from progressing to a proangiogenic state. In conclusion, several PK/PD factors contributing to the antitumor activity of the MD TMZ therapy have been identified and form a foundation for further investigations of low-dose TMZ regimens.

Gliomas represent the most common and aggressive type of primary brain tumors, accounting for 42% of all primary nervous system tumors and 77% of all malignant primary central nervous system tumors (Ashby and Ryken, 2006). Despite advances in the multidisciplinary approach and diagnostic imaging techniques, the overall prognosis for patients with high-grade malignant gliomas still remains bleak, with a median survival measured in months and an overall 5-year survival rate of ≤2% (Surawicz et al., 1998). In the past, the chemotherapeutic approach to malignant brain tumors relied mainly on the use of nitrosoureas, which showed only modest antitumor activity (Nieder et al., 2000). More recently, 8-carbamoyl-3-methylidazo(5,1-d)-1,2,3,5-terrazin-4(3H)-one (TMZ, temozolomide) has emerged as a well-tolerated oral alkylating agent that is able to cross the blood-brain barrier with demonstrated encouraging activity in primary and recurrent gliomas (Newlands et al., 1992; Dehdashti et al., 2006).

TMZ is rapidly and well absorbed after oral administration (Stevens et al., 1987; Newlands et al., 1992) and undergoes spontaneous hydrolysis at physiological pH to form its active metabolite, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC), which further degrades to 5(4)-aminomidazole-4(5)-carboxamide and a highly reactive methyl-diazonium cation (Stevens et al., 1987; Denny et al., 1994). MTIC exerts its cytotoxic effect primarily through methylation of genomic DNA at the O6 position of guanine. The formation of O6-methylguanine (O6MeG) subsequently induces futile cycling of a mismatch repair pathway, leading to inhibition of DNA replication and cell-cycle arrest at the G2-M phase transition (Denny et al., 1994; Roos et al., 2007). The O6MeG lesion can...
be repaired by cellular O6-methylguanine-DNA methyltransferase, which transfers the methyl group to a cysteine residue (Trivedi et al., 2005).

The standard recommended dose of TMZ as a single agent or in combination is 150 to 200 mg/m² daily for 5 consecutive days repeated every 4 weeks (Brandes et al., 2002). Other administration schedules, including compressed and extended dosing protocols, evaluated with the intention of maximizing O6-methylguanine-DNA methyltransferase depletion and hence potentiating the cytotoxicity, have demonstrated limited improvement of clinical efficacy with potentially increased hematological toxicity (Danson et al., 2003; Tosoni et al., 2006). Thus, there is a need to reappraise the dose and schedule of TMZ to further improve its effectiveness while reducing its toxicity.

Metronomic chemotherapy, which refers to the frequent administration of chemotherapeutics at doses significantly below the maximum tolerated dose without prolonged drug-free breaks, has been evaluated as an alternative strategy to achieve long-term therapeutic control (Gasparini, 2001; Kerbel and Kamen, 2004). The primary mechanism for metronomic chemotherapy is thought to be inhibition of tumor angiogenesis by direct killing of endothelial cells in the tumor neovasculature (Miller et al., 2001) and suppressing the mobilization and levels of bone marrow-derived circulating endothelial progenitor cells (Bertolini et al., 2003). As such, metronomic chemotherapy may possess several advantages over conventional chemotherapy, including delaying the onset of acquired drug resistance and reducing host toxicity. Two milestone studies by Browder et al. (2000) and by Klement et al. (2000) followed by several subsequent studies have illustrated the capacity of different cytotoxic agents to preferentially target the endothelial cell compartment of tumors when lower doses were given more frequently (Shaked et al., 2005; Klink et al., 2006).

Several studies have been carried out to assess the antiangiogenic potential of the protracted low-dose TMZ treatment. An early preclinical study demonstrated that angiogenesis was inhibited by 5 μM TMZ in both choroidal neovascular membrane and human umbilical vein endothelial cell (HUVEC)-based Matrigel assays (Kurzen et al., 2003). A recent clinical study showed that after receiving continuous low-dose TMZ plus rofecoxib treatment, patients harboring highly angiogenic glioblastoma multiforme had a significantly longer time to progression compared with patients with low angiogenic glioblastoma multiforme (Tuettenberg et al., 2005), which suggested an antiangiogenic activity for continuous low-dose TMZ plus rofecoxib. A more recent preclinical study demonstrated that metronomic treatment with TMZ exerted both antiangiogenic and antitumor effects in a TMZ-resistant C6/LacZ rat glioma model (Kim et al., 2006).

Although previous studies showed that TMZ might possess potential antiangiogenic properties when given metronomically, further investigation is warranted to understand PK-PD relationships that can guide the selection of optimal metronomic dosing regimens of TMZ. Therefore, the aim of the present study was to characterize and contrast the pharmacokinetics and pharmacodynamics of TMZ in nude rats with human glioma xenografts receiving either CD or MD TMZ treatment, and in so doing, provide a foundation for the design of optimal MD TMZ treatments.
using the following formula: \( V = 0.5a \cdot b^2 \) (Kato et al., 1994). At a tumor size of \( -1 \) cm\(^3\), nude rats were randomized into three groups: 1) the vehicle control group (daily i.v. administration of 25% dimethyl sulfoxide in saline), 2) the CD group (daily i.v. administration of TMZ for 5 days at a dose of 18 mg/kg by a 10-min infusion at a rate of 2 mg/kg/min), and 3) the MD group (daily i.v. bolus injection of TMZ for up to 28 days at a dose of 3.23 mg/kg/day). The dosing regimen of TMZ for the CD group is equivalent to the TMZ dose of 200 mg/m\(^2\)/day for 5 days used clinically on the basis of the reported formula of conversion between rat body weight and surface area (Freireich et al., 1966). A course of MD TMZ treatment consisted of daily administration of 3.23 mg/kg TMZ for 28 days, which corresponded to a cumulative dose of 90 mg/kg and was equivalent to the total of the five daily treatments of 18 mg/kg TMZ of the CD regimen. On days 5, 14, and 28, a subgroup of rats from each group was euthanized with CO\(_2\), and the tumor mass was excised. The tumor was dissected using a razor blade, snap-frozen in liquid nitrogen, and then stored at \(-80^\circ\)C before total RNA extraction and tissue lysate preparation. Body weights and tumor volumes were measured twice a week throughout the experiment.

**Measurement of Tumor pH and IFP.** Tumor pH and IFP in both the central and peripheral regions of the tumor were measured 1 day before the initiation of both CD or MD TMZ treatment and once a week thereafter. The extracellular pH of tumor tissue was measured by inserting a small needle probe connected to a pH meter (Jencons Electronics Ltd., San Diego, CA) into the tumor. The measurement of IFP was performed using the “wick-in-needle” technique that also entailed the placement of a small probe connected to a pressure transducer (Kent Scientific Co., Torrington, CT) into the tumor (Fadnes et al., 1977).

**PK Blood Sampling and Tumor Microdialysis Study.** One day before the initiation of TMZ treatment, a right femoral vein and femoral artery were surgically catheterized for drug administration and blood sampling, respectively. The pharmacokinetics of TMZ was assessed in two separate groups of seven to nine rats, which were given either daily i.v. administration of 18 mg/kg TMZ by a 10-min infusion at a rate of 2 mg/kg/min for 5 days (the CD group) or a daily IV bolus injection of 3.23 mg/kg TMZ for 28 days (the MD group). PK blood sampling periods were on both the first and last days (day 5 and day 28 for CD and MD groups, respectively) of dosing with 100-\(\mu\)l blood samples collected from each rat at predose, 5, 10, 20, and 40 min, and 1, 1.5, 2, 3, 4, 5, 6, and 8 h after the start of drug administration. The blood samples were immediately centrifuged, and 50 \(\mu\)l of plasma was acidified with 10 \(\mu\)l of 0.1% formic acid and added to 100 \(\mu\)l of acetonitrile to precipitate proteins. After centrifugation at 14,000 rpm for 5 min, the supernatant was collected and stored at \(-80^\circ\)C until analysis.

On the day of the experiment, CMA/20 dialysis probes with a membrane length of 10 mm were inserted into the central region of the tumor. Before the administration of TMZ, the probes were perfused at a rate of 2 \mu\l/min with Ringer’s solution (147 mM NaCl, 2.3 mM CaCl\(_2\), and 4.0 mM KCl) containing 200 ng/ml TMZ. After a 45-min equilibration period, microdialysate samples were collected automatically with a CMA/170 refrigerated fraction collector for 40 min in fractions corresponding to 10-min intervals. The in vivo recovery determined from the loss of TMZ from the perfusate was calculated for each interval of time according to the following equation (Wang et al., 1993):

\[
RR = \frac{C_{in} - C_{out}}{C_{in}}
\]

where \(RR\) is the in vivo relative recovery, \(C_{in}\) is the drug concentration in perfusate, and \(C_{out}\) is the drug concentration in the microdialysate. The mean \(RR\) value obtained from four individual determinations was used as the in vivo recovery to correct the dialysate concentrations that allowed unbound TMZ concentrations in the interstitial fluid to be calculated as the concentrations in the microdialysate divided by the mean \(RR\).

After the calibration period for the microdialysis probes, a washout period of 40 min with blank Ringer’s solution perfusion was allowed before i.v. TMZ administration. Microdialysate samples were collected at 10-min intervals for 6 h after TMZ administration at a flow rate of 2 \mu\l/min and then stored at \(-80^\circ\)C until analysis.

**Drug Analysis.** TMZ concentrations in the plasma and tumor microdialysate were quantitated using electrospray ionization LC/MS/MS. In brief, the plasma sample (50 \(\mu\l\)) with the addition of the internal standard dacarbazine (8 ng/ml) was deproteinized with acetonitrile containing 0.1% formic acid (100 \(\mu\l\)). After centrifugation (14,000 rpm for 10 min), an aliquot of 7 \(\mu\l\) of the supernatant was injected into the LC/MS/MS system. An aliquot of 15 \(\mu\l\) of tumor microdialysate spiked with the internal standard (17 ng/ml) was directly injected into the LC/MS/MS system. The LC system consisted of a C\(_{18}\) guard cartridge (4.0 × 2.0 mm; Phenomenex, Torrance, CA) and a Luna C\(_{18}\) analytical column (50 × 2.0 mm, 3 \(\mu\)m particle size; Phenomenex) with an isotropic mobile phase consisting of acetonitrile-0.5 mM ammonium formate containing 0.1% formic acid (8:92 and 4:96, v/v, for plasma and microdialysate samples, respectively). The flow rate was 0.2 ml/min. The column temperature was maintained at 35°C. The instrument was operated in the positive ion scan mode, monitoring the ion transitions from m/z 195.1 → 137.9 for TMZ and m/z 183.2 → 123.3 for dacarbazine (the internal standard) with a dwell time of 800 ms for each ion transition. The lower limits of quantification were 9 and 8 ng/ml for TMZ in plasma and tumor microdialysate, respectively. The mean extraction recoveries of TMZ were 103.6 and 94.8% for plasma and tumor microdialysate, respectively.

**PK Analysis.** PK data analyses were performed using the software package SAAM II (version 1.2; University of Washington, Seattle, WA). A hybrid PK model that consisted of a forcing function describing the plasma concentration-time profile and a one-compartment tumor model was used to characterize drug disposition in plasma and tumor tissue (Fig. 1). Parameters estimated in the model included the volume of distribution (\(V_p\)), the elimination rate constant (\(K_p\)), the intercompartmental rate constants (i.e., \(K_{pt}\) and \(K_{tp}\)), and the area under the plasma or tumor IF concentration-time curve (AUC\(_{0→t}\) and AUC\(_{0→t}\) for plasma and tumor IF, respectively) from 0 to the last quantifiable time point. The volume of the tumor compartment (\(V_t\)) was fixed at the actual tumor volume measured in

![Fig. 1. Schematic presentation of the PK model containing a two-compartment plasma disposition model and a one-compartment tumor model. Model parameters include the volume of distribution of the plasma compartment (\(V_p\)), the elimination rate constant (\(K_p\)), and the intercompartmental rate constants for unbound TMZ (i.e., \(K_{pt}\) and \(K_{tp}\)). The unbound fraction of TMZ in plasma (\(f_u\)) is 0.8 (Gallo et al., 2004). The volume of the tumor compartment (\(V_t\)) was fixed at the actual tumor volume measured in individual animals on the day of PK sampling. *+, the two-compartment model is shown for the plasma compartment, yet some animals were best-characterized by a one-compartment model.](image-url)
individual animals on the day of PK sampling. PK parameters calculated from these estimates included systemic clearance (CLs), and the total area under the concentration-time curves (AUC0–∞). The peak plasma concentration (Cmax) and the peak time (tmax) were obtained by visual inspection of the plasma and tumor IF concentration-time curves.

Quantitative Real-Time PCR Assay. One microgram of total RNA extracted from tumor tissues with TRIzol reagent was reverse-transcribed into complimentary DNA (cDNA) using random hexamer primers and avian myeloblastosis virus reverse transcriptase reagents as per the manufacturer's protocol (Promega, Madison, WI). Then, 50 ng of cDNA was subjected to quantitative real-time PCR for the selected genes, i.e., human VEGF, rat HIF-1α, rat TSP-1, rat Ang-1, rat Ang-2, rat Tie-2, and human β-actin (the endogenous control), using the predeveloped 20× TaqMan gene expression assay mix (assay identification numbers are Hs00173626_m1, Rn00577560_m1, Rn00449032_m1, Rn00585552_m1, Rn01433371_m1, and Hs99999903_m1 for human VEGF, rat HIF-1α, rat TSP-1, rat Ang-1, rat Ang-2, rat Tie-2 and human β-actin, respectively; Applied Biosystems).

PCR amplification was performed on 2 μl of cDNA template in a 25-μl reaction mixture containing 12.5 μl of TaqMan universal PCR master mix, 1.25 μl of TaqMan gene expression assay mix (Applied Biosystems), and 9.25 μl of RNase-free water. During the extension phase of PCR, the nucleolytic DNA polymerase cleaved the hybridization probe, and the resulting relative increase in the reporter fluorescent dye emission was monitored in real time using a 7300 real-time PCR system (Applied Biosystems). The fluorescent dye emission was a function of cycle number and was determined using the sequence detection software (Applied Biosystems), giving the threshold cycle number (Ct) at which the increase in normalized fluorescence over a defined threshold that first occurs for each amplification plot. Each RNA sample was tested in quadruplicate, and the Ct values were averaged. The relative amounts of the six genes were calculated by means of the ΔΔCt method. In brief, the arithmetic formula for the ΔΔCt method is given by $2^{-\Delta\Delta Ct}$, where ΔCt is the Ct value of each target gene minus the Ct value of the β-actin, which gives a normalized value. The ΔΔCt was obtained by subtracting the normalized ΔCt value of each target gene from the normalized ΔCt value of a calibrator, which was a tumor sample obtained from an untreated rat (neither vehicle nor TMZ). The formula $2^{-\Delta\Delta Ct}$ therefore gives a relative value when the target is compared with the calibrator.

Western Blot Analysis. Tumor tissues were homogenized in 1 ml of cell lysis buffer (50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 25 mM β-glycerophosphate, 50 mM NaF, 10 mM Na3VO4, and protease inhibitor cocktail) per 200 μg of frozen tissue. Lysis was carried out at 4°C for 30 min, and lysates were centrifuged at 15,000 rpm for 20 min. The protein concentration of the supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad). Equal amounts (70 μg) of proteins were separated on precast sodium dodecyl sulfate-polyacrylamide gels (Life Therapeutics, Frenchs Forest, Australia) and transferred to polyvinylidene difluoride membranes. After being blocked in Tris-buffered saline with 0.1% Tween 20 containing 5% nonfat milk, the membranes were immunoblotted overnight at 4°C with the following antibodies: VEGF (1:400), HIF-1α (1:400), TSP-1 (1:400), Ang-1 (1:400), Ang-2 (1:200), and Tie-2 (1:400). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:15,000; Santa Cruz Biotechnology) at room temperature for 1 h, and immunoreactive protein bands were visualized by the enhanced chemiluminescence system (PerkinElmer Life and Analytical Sciences). The membranes were then stripped and reprobed with β-actin (1:4000) to ensure equal protein loading. Band areas were quantified by ImageJ software (from National Institutes of Health and available at http://rsb.info.nih.gov/ij/). The expression levels of individual proteins are presented as fold changes compared with the nontreated control (i.e., the same tumor sample used as the calibrator for the real-time PCR).

Results

In Vitro Cytotoxicity of TMZ in HUVECs and SF188+ Cells. The therapeutic action of anticancer drugs may be attributed to toxicity to both proliferating endothelial cells and tumor cells (Gasparini, 2001; Hahnfeldt et al., 2003). On the basis of previous observations that various cytotoxic drugs acted preferentially on endothelial cells when the cells were exposed to the individual drugs daily for up to
6 days (Bocci et al., 2002), we contrasted the in vitro cytotoxicity of protracted TMZ exposure for 144 h to the relatively short TMZ exposure for 24 h in HUVECs and SF188V+ human glioma cells to determine whether MD TMZ treatment could have the same propensity to inhibit endothelial cell growth. PAC, which has consistently shown preferential antiproliferative activity against endothelial cells when given at low doses in a continuous manner, served as a positive control in this study (Bocci et al., 2002). In addition, standard culture conditions were used in this study to mimic certain aspects of the in vivo tumor microenvironment by adding several proangiogenic growth factors in the culture medium. When SF188V+ glioma cells and HUVECs were exposed to TMZ or PAC for only 24 h, both drugs displayed moderate or mild cytotoxic properties with the IC₅₀ values of >1 nM for TMZ in both cell lines, whereas for PAC the IC₅₀ values were 1.79 nM and >3 μM in SF188V+ cells and HUVECs, respectively (Fig. 2). In sharp contrast, protracted low-dose TMZ and PAC treatment for 144 h significantly inhibited the growth of SF188V+ cells and HUVECs at relatively low concentrations compared with the 24-h treatment. The IC₅₀ values for SF188V+ cells and HUVECs in the 144-h TMZ treatment were ~48- and 39-fold lower, respectively, than those in the 24-h treatment (P < 0.01). Likewise, the IC₅₀ value for SF188V+ cells in the 144-h PAC treatment was 2.6-fold less than that in the 24-h treatment (P < 0.01).

Notably, the cytotoxicity of TMZ appeared to be more pronounced against SF188V+ cells than against HUVECs (IC₅₀ of 24.0 ± 0.51 versus 35.8 ± 1.15 μM, P < 0.01). In contrast, the control drug PAC was in agreement with that in the literature (Bocci et al., 2002), showing a more potent cytotoxic effect on HUVECs than on SF188V+ cells (IC₅₀ of 0.680 ± 0.048 versus 0.288 ± 0.037 nM, P < 0.01).

**Systemic and Tumor Distribution of TMZ in Tumor-Bearing Athymic Rats.** The PK study was designed to compare the disposition of TMZ in plasma and tumors after systemic and tumor IF in SF188V+ tumor-bearing athymic rats receiving multiple i.v. administrations of TMZ at a dose level of either 18 mg/kg (CD regimen) or 3.24 mg/kg (MD regimen) for this purpose, microdialysis was used so that interstitial fluid unbound drug concentrations could be measured, this extravascular sampling compartment being adjacent to both tumor and endothelial cells. Seven rats from the CD group and nine from the MD group had complete PK studies performed on the 1st day and the last day of treatment. A compartmental modeling analysis applied to individual rats in the CD and MD groups revealed that for some animals the best fit of plasma concentration data was achieved with a one-compartment model, whereas a two-compartment model provided the best fit for the others. Once the best-fit compartment model was defined for each animal from the plasma-time profile of TMZ, it was set as a forcing function in the compartment model that was fit to the corresponding TMZ tumor concentrations (Fig. 1). Definition of a constant forcing function in the development of the tumor models minimized the number of parameters that had to be estimated. The PK data for TMZ at each dosing regimen are summarized in Table 1, with mean plasma and tumor IF TMZ concentration-time profiles as well as the representative predicted and observed plasma and tumor IF concentrations shown in Fig. 3, A through D. Compartmental model-predicted TMZ concentrations in both plasma and tumor IF showed remarkably good agreement between predicted and observed values (Fig. 3, C and D). The pharmacokinetics of TMZ appeared linear, both dose- and time-independent, as there were no differences between the systemic clearance and volume of distribution in the CD and MD groups on day 1 and the last treatment day. Within each type of TMZ dosing schedule, these parameters remained essentially constant on day 1 and day 5 in the CD group and on day 1 and day 28 in the CD group. The ratio of the mean AUC₀₋→ₚ values on day 1 in the CD group to those in the MD group was 5.6, which was identical to the dose ratio of 5.6 for the CD to MD groups.

**TABLE 1**

PK parameters of TMZ in plasma and tumor IF in SF188V+ tumor-bearing athymic rats receiving multiple i.v. administrations of TMZ at a dose level of either 18 mg/kg (CD regimen) or 3.24 mg/kg (MD regimen).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CD (n = 7)</th>
<th>MD (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘₐₓₚ (µg/ml)</td>
<td>24.1 ± 3.7</td>
<td>21.6 ± 3.2</td>
</tr>
<tr>
<td>AUC₀₋ₚ (µg·h/ml)</td>
<td>24.1 ± 2.6</td>
<td>25.0 ± 5.7</td>
</tr>
<tr>
<td>Vₚ (l/kg)</td>
<td>0.91 ± 0.30</td>
<td>0.98 ± 0.44</td>
</tr>
<tr>
<td>CLₚ (l/h/kg)</td>
<td>0.73 ± 0.11</td>
<td>0.75 ± 0.15</td>
</tr>
<tr>
<td>K₁₋₂ (h⁻¹)</td>
<td>0.87 ± 0.26</td>
<td>0.86 ± 0.29</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>0.85 ± 0.20</td>
<td>0.89 ± 0.28</td>
</tr>
<tr>
<td>Tumor IF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cₘₐₓₜ (µg/ml)</td>
<td>10.6 ± 3.6</td>
<td>11.1 ± 5.9</td>
</tr>
<tr>
<td>tₐₘₐₓₜ (h)</td>
<td>0.51 ± 0.21</td>
<td>0.77 ± 0.22</td>
</tr>
<tr>
<td>AUC₀₋ₜ (µg·h/ml)</td>
<td>21.0 ± 5.4</td>
<td>31.7 ± 15.1</td>
</tr>
<tr>
<td>Vₜ (ml)</td>
<td>1.22 ± 0.30</td>
<td>1.33 ± 0.32</td>
</tr>
<tr>
<td>AUC₀₋ₜ/AUC₀₋ₚ ratio</td>
<td>0.90 ± 0.35</td>
<td>1.22 ± 0.49</td>
</tr>
<tr>
<td>Intercompartmental rate constant Kᵣ (h⁻¹)</td>
<td>4.30 ± 5.00</td>
<td>2.69 ± 1.30</td>
</tr>
<tr>
<td>Kᵣ (h⁻¹)</td>
<td>3.02 ± 2.28</td>
<td>1.88 ± 1.47</td>
</tr>
</tbody>
</table>

*P < 0.01 comparing with the CD group on day 5 using the Wilcoxon rank-sum test.
In addition, as expected from the systemic clearance and volume of distribution values, the $t_{1/2}$ of TMZ remained essentially the same at $\sim 0.9$ h, independent of the dose (CD and MD groups) and time (1st day and last day of treatment).

The analyses of the pharmacokinetics of TMZ in tumor indicated trends similar to those in plasma on the basis of the CD and MD doses and study days with the exception of the more rapid distribution to the tumor tissue ($K_{pt}$) in the MD group compared with that in the CD group (6.77 versus 2.69; $P < 0.01$) on the last day of treatment (Table 1). There was no significant difference in the time of peak tumor concentrations between the CD and MD groups. The unbound TMZ concentrations in tumor decreased rapidly thereafter with $t_{1/2}$ values similar to those determined in plasma and ranged from a mean of 0.99 to 1.28 h. The CD/MD day 1 ratios of both $C_{max}$ and $AUC_{tumor}$ values were 2.2 and 3.2, respectively, and less than those anticipated from the dose ratio of 5.6. This potential nonlinear phenomenon was not observed on the last treatment days (days 5 and 28) when the $C_{max,t}$ and $AUC_{tumor,t}$ ratios were 5.5 and 6.7, respectively, analogous to the dose ratio of 5.6. The lower than expected ratios of $C_{max}$ and $AUC_{tumor}$ on day 1 could be attributed to the day 1 TMZ measurements in the MD treatment group in which TMZ concentrations were higher than those obtained on day 28. These high TMZ measurements also translated into higher than expected mean $AUC_{tumor}/AUC_{plasma}$ ratios in the MD group on day 1, being 1.7-fold higher than that in the CD group, although the difference was not statistically significant ($P > 0.05$). However, on the last day of treatment (day 5 for CD and day 28 for MD), the mean $AUC_{tumor}/AUC_{plasma}$ ratios were analogous at a mean of 1.25. In considering TMZ PK data over the complete treatment periods, there were no sustainable changes in tumor accumulation between the CD and MD regimens, consistent with the relatively short half-life ($\sim 0.9$ h) and linear PK behavior of the drug.

### Toxicity

Because there were no animal deaths in this study, animal weight loss was used as an indicator of toxicity associated with either the CD or MD TMZ treatment. One week after the start of the treatment, weight loss was observed in treated animals, but it was only 9 and 6% for the CD and MD groups, respectively. The weights of animals receiving both CD and MD TMZ treatments were regained by the end of the 28-day study period. An analysis of variance showed a lack of statistically significant differences in body weight between control and TMZ treatment groups before and during the period of treatment ($P > 0.05$). Except for...
suppressing body weight gain in rats, no other systemic toxicity related to the MD TMZ treatment was observed, demonstrating that the MD regimen applied in this study is well tolerated in the rat model.

Effect of TMZ on SF188V+ Tumor Growth, Tumor pH, and IFP in Nude Rats. Tumor size is the most common parameter used to assess effective anticancer treatment in preclinical models. Although considered a crude endpoint, it is important to include its measurement with the hope of identifying correlations to PK/PD measurements that could serve as the basis for designing drug dosing regimens. As expected, SF188V+ xenografts grew progressively in the vehicle control animals with a 3-fold increase in the mean tumor volume by day 28 ($P < 0.01$) (Fig. 4A). In contrast, tumors treated with either CD or MD TMZ approached a highly significant delay in tumor growth compared with the control, with maximal tumor growth inhibitions of 71 and 49% for the CD and MD groups, respectively ($P < 0.01$) (Fig. 4A). Notably, tumor growth delay in the CD group persisted for up to 3 weeks after the cessation of the treatment. In addition, there was no statistical difference in tumor size between the CD and MD groups.

Tumor IFP is used as an indicator of vascular integrity and hypoxia and often shows regional variations within the tumor (Jain, 1987). Large tumors containing regions of hypoxia will possess higher IFPs than smaller well perfused tumors. These types of changes can influence macromolecular and small molecular weight drug transport by convection. Consistent with that expectation for large untreated tumors, we found that IFP was significantly increased in the control group on days 21 and 28 irrespective of the region of the tumor ($P < 0.01$ for both). Both CD and MD TMZ treatments produced a significant decrease in IFP in both the central and peripheral regions of the tumor compared with the control from day 7 through day 28 (Fig. 4B). These data support the possibility that TMZ could enter tumors by convective transport because of the reduction in IFP.

Tumor cells have a natural tendency to overproduce acids, resulting in very acidic pH values in the extracellular space. This acidity can be further exacerbated by oxygen depletion and nutrient/energy deprivation in large tumors (Vaupel et al., 1989). Consistent with these findings, our data showed that tumor pH in the control group tended to decline as the tumor volume increased regardless of the region of the tumor, and on day 28, a statistically significant decrease in tumor pH was observed in the central region of tumors ($P < 0.05$), whereas a trend toward lower pH values was found for the peripheral region ($P = 0.069$) (Fig. 4C). Tumor pH may affect the formation of MTIC, which usually results from the spontaneous hydrolysis of TMZ at physiological pH (Stevens et al., 1987; Denny et al., 1994). However, we observed that tumor pH in the TMZ treatment groups basically remained unchanged and tended to be greater than that in the control groups with the only significant difference being found on day 28 when tumor pH was greater (mean values $-7.15$) in the MD group but not in the CD group compared with control. In this regard, the effect of tumor pH on the cytotoxicity of TMZ related to the formation of MTIC would be marginal.

Evaluation of mRNA Expression Levels of VEGF, HIF-1α, TSP-1, Ang-1, Ang-2, and Tie-2 in SF188V+ Xenografts. Tumor-induced angiogenesis is essential for the progression and metastasis of solid tumors. This process...
involves a diverse array of molecules, including those that stimulate endothelial cell proliferation and migration as well as those that regulate the maintenance and destruction of the perivascular milieu. To characterize the angiogenic-related molecular and cellular responses that occur after CD and MD TMZ treatment, we examined changes in expression of several angiogenic mediators, VEGF, HIF-1α, TSP-1, Ang-1, Ang-2, and Tie-2, in response to the CD and MD regimens using quantitative real-time PCR.

No significant changes in VEGF mRNA expression levels were observed in the control group during the experimental period. This finding could be attributable to the high basal expression level of the VEGF gene in SF188V+ cells, which were designed to overexpress VEGF. The observed elevation of the HIF-1α gene expression level in the control group on day 5 compared with the basal level is consistent with the anticipation that rapid tumor growth results in oxygen depletion, leading to the up-regulation of HIF-1α expression, whereas the significantly decreased mRNA expression level of HIF-1α by day 28 (P < 0.05) suggests that tumor cells have adapted to survive under conditions of hypoxia. Interestingly, the expression of VEGF mRNA was significantly up-regulated in both CD and MD groups by day 14 (P < 0.05 for both), but fell below the respective median VEGF expression levels observed on day 5 by day 28. Likewise, HIF-1α gene expression levels in both treatment groups were increased by day 14 followed by a significant decrease by day 28 (P < 0.05 for both TMZ treatment groups) (Table 2). Significant correlations between VEGF and HIF-1α gene expression were demonstrated in the correlative plots of \(2^{-\Delta\Delta C_t}\) values of VEGF (y-axis) versus HIF-1α (x-axis) obtained from matched samples, with Pearson correlation coefficients of 0.49 (P = 0.018), 0.68 (P = 0.003), and 0.84 (P = 0.000002) for the control, CD, and MD groups, respectively (Fig. 5). This observation is consistent with the expected up-regulation of VEGF in response to hypoxia. There was no clear trend in the effect of either CD or MD TMZ treatment on the expression levels of TSP-1, Ang-1, Ang-2, and Tie-2 genes in SF188V+ xenografts, and, further, no significant correlations were noted among any other gene expression values in any of the study groups, suggesting that multiple mechanisms and pathways are involved in tumor angiogenesis.

**Western Blot Analysis.** In addition to transcriptional changes in genes associated with selected PD markers of angiogenesis, it was important to also determine protein expression of the same markers as they may not be correlated and have a different time profile. Because protein expression was monitored at multiple time points during the 28-day study period, there was an opportunity to identify time-dependent changes in protein induction and inhibition. Semi-quantitative analyses of the expression levels of VEGF and HIF-1α protein were basically in agreement with the real-time PCR data, although the fold change in the mRNA expression level was not proportionally reflected at the protein level (Fig. 6, A–C). Western blot analysis showed that VEGF expression levels were slightly increased in the control and MD groups (118.1 ± 33.8 and 122.1 ± 32.2% of the nontreated control, respectively). In contrast, there was a reduction of up to 36% of the basal level of VEGF expression in the MD group with a trend toward significance compared with the control.

### Table 2

Effect of conventional and metronomic TMZ treatment on relative mRNA expression levels of VEGF, HIF-1α, TSP-1, Ang-1, Ang-2, and Tie-2 in SF188V+ tumors (n = 7–8)

<table>
<thead>
<tr>
<th>Gene/Treatment Group</th>
<th>Gene Expression Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>VEGF</td>
<td>2.18 (1.29–46.97)</td>
</tr>
<tr>
<td>Control</td>
<td>13.55 (6.95–34.63)</td>
</tr>
<tr>
<td>CD</td>
<td>9.39 (2.38–65.79)</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>4.54 (0.44–22.07)</td>
</tr>
<tr>
<td>Control</td>
<td>1.96 (1.08–4.31)</td>
</tr>
<tr>
<td>CD</td>
<td>2.06 (0.92–10.09)</td>
</tr>
<tr>
<td>MD</td>
<td>118.1 ± 33.8</td>
</tr>
</tbody>
</table>

* P < 0.05 comparing with the same treatment group on day 5 using Wilcoxon rank-sum test; † P < 0.05 comparing with the same treatment group on day 14 using Wilcoxon rank-sum test.

**Fig. 5.** Scatter plots of the HIF-1α gene expression increase with the VEGF gene expression in SF188V+ tumors obtained from the (A) control group, (B) conventional TMZ treatment group, and (C) metronomic TMZ treatment group. There was a significant correlation between HIF-1α and VEGF gene expression in all three groups.
group on day 28 ($P = 0.066$) (Fig. 6B). A significant up-regulation of HIF-1α expression was observed in the control group on day 5 ($P < 0.05$ and $0.01$ compared with the CD and MD groups, respectively) and in the CD group on day 14 ($P < 0.05$ compared with either the same treatment group on day 5 or the MD group on day 14). The expression levels of HIF-1α in both CD and MD groups declined significantly on day 28 compared with those in the respective groups on day 14 ($P < 0.05$ and $0.01$ for the CD and MD groups, respectively), and a significant difference was also found between the control and MD groups on day 28 ($125.8 \pm 29.2$ versus $87.2 \pm 30.5$, $P < 0.05$) (Fig. 6C). Overall, similar to our real-time PCR findings, better control of angiogenic factors seems to be maintained with the MD regimen (i.e., avoidance of larger fluctuations) than with the CD regimen. No significant changes in expression of selected angiogenic mediators other than VEGF and HIF-1α were observed among the control and two TMZ treatment groups at any time point (data not shown), suggesting that MD TMZ does not act on any specific pathways mediating angiogenesis as do other MD chemotherapeutic agents, such as cyclophosphamide and PAC (Damber et al., 2006).

### Discussion

MD chemotherapy has recently received a great deal of attention as an alternative strategy to conventional maximum tolerated dose regimens for cancer management because its use may potentially avoid or at least delay the onset of chemoresistance by targeting tumor vasculature (Gasparini, 2001; Kerbel and Kamen, 2004). Although recent studies have demonstrated that angiogenesis can be inhibited by low concentrations of TMZ as effectively in vitro as in vivo (Kurzen et al., 2003; Kim et al., 2006), a better understanding of PK and PD differences between conventional and metronomic TMZ therapy may lead to development of more effective and less toxic therapeutic regimens.

The results of the in vitro cytotoxicity assay revealed that the antiproliferative effect of TMZ on both SF188V+ cells and HUVECs was greatly increased when cell proliferation was assessed under protracted treatment conditions in which the drug-containing medium was replaced on a daily basis, although the fold changes in the IC50 values between the 24- and 144-h treatment might be exaggerated because of different starting cell concentrations as well as the potentially delayed cytotoxic effect of TMZ. Nonetheless, the results suggest that TMZ is increasingly cytotoxic with increasing time of exposure. An intriguing observation was that extending exposure time increased the antiproliferative potency of TMZ in SF188V+ cells and HUVECs to the same extent, suggesting that the antiproliferative effects of TMZ on both tumor and endothelial cells are essentially identical. The observed statistically significant difference in IC50 values between SF188V+ cells and HUVECs, however, could be due to the faster growth of tumor cells in culture compared with that of endothelial cells as the rates of DNA replication and cell proliferation are known to be essential determinants for TMZ-induced O6MeG-triggered cell killing (Denny et al., 1994, Roos et al., 2007). Although an early study by Kurzen et al. (2003) demonstrated that TMZ reduced the adhesion of HUVECs to fibronectin and inhibited endothelial cell align-
ment and cord formation on Matrigel when HUVECs were exposed to TMZ at 5 to 50 \( \mu M \) for 24 h, it could be possible that the inhibitory effect of TMZ on endothelial cell functions relevant to angiogenic process was associated with its anti-proliferative activity. Therefore, TMZ would have both cytotoxic and antiangiogenic activity in vivo at doses that inhibit the proliferation of both tumor and endothelial cells.

A comparison between the in vivo drug levels and in vitro IC\(_{50}\) values for the 144-h treatment in this study revealed that the in vivo tumor IF unbound TMZ concentrations in the CD group were over the IC\(_{50}\) values for SP188V+ cells and HUVECs for \(~3\) h/day, whereas those in the MD group were far less than the IC\(_{50}\) values. However, the effectiveness of MD TMZ was found to be comparable to that of the CD regimen in terms of inhibition of tumor growth and decreased tumor IFP and pH relative to the control group. It was therefore speculated that TMZ might have affected tumor-induced angiogenesis in vivo at local concentrations lower than those necessary to cause a cytotoxic effect on tumor cells, and the antiangiogenic activity of TMZ at low doses might be attributed to mechanisms other than direct inhibition of endothelial cell proliferation.

To explore whether MD TMZ may exert the in vivo tumor suppressive effect through an antiangiogenic mechanism, we investigated the effects of different TMZ dosing regimens on the expression of several cytokines and their receptors that are known to play a pivotal role in tumor angiogenesis. VEGF gene expression levels in both CD and MD groups were found to be higher than that in the control group by day 14. A significant correlation between the mRNA expression level of VEGF and that of HIF-1\(\alpha\) suggests that VEGF gene expression level is elevated in response to hypoxia. Although no significant changes in VEGF protein expression levels were observed by Western blot analyses, the protein expression level of HIF-1\(\alpha\) in the CD group was significantly up-regulated by day 14, whereas the HIF-1\(\alpha\) protein expression levels in the MD group remained consistent throughout the treatment period, implying that the effect of TMZ on hypoxia could be dose-related. Although the exact mechanism of the exacerbated hypoxia after TMZ treatment deserves further investigation, the destructive action of TMZ, especially at higher doses, on the tumor vasculature by direct killing of endothelial cells may have caused hypoxia, leading to the initial elevation of HIF-1\(\alpha\) and VEGF gene expression levels. No clear trend could be discerned for the effect of both the CD and MD TMZ regimen son the expression of TSP-1, Ang-1, Ang-2, and Tie-2 in this study. Taken together, our results suggest that the antitumor activity of TMZ at either high or low dose levels may be attributed to its antiproliferative activity against both tumor cells and endothelial cells. Nonetheless, unlike the CD treatment that may result in a temporary excessive vascular regression and subsequent exacerbation of local hypoxia in tumors, the moderate effect of the MD treatment on tumor endothelial cells may lead to the normalization of tumor vasculature. This in turn could prevent tumors from progressing to a proangiogenic state that may lead to the enhanced invasiveness of the tumor and an increased possibility of metastatic dissemination (Le et al., 2004).

It is noteworthy in the PK study that, unlike the plasma pharmacokinetics of TMZ that was demonstrated to be linear, both as a function of dose and time, tumor uptake of TMZ was less consistent and suggested a possible nonlinear phenomenon in the rat model. The mechanisms underlying this potential nonlinear tumor distribution are open to speculation. TMZ is thought not to undergo any specialized membrane transport processes but to enter and exit cells via passive diffusion because of its high lipophilicity (Bull and Tisdale, 1987). Given an intact tumor microvascular structure, unbound or protein-free drug is available for diffusion because of a concentration-gradient across the cell membrane. In this case, the tumor interstitial fluid unbound TMZ/total plasma AUC ratios (AUC\(_{\text{tumor}}/\text{AUC}_{\text{plasma}}\)) should be on the order of 0.8, which is equivalent to the unbound fraction of TMZ determined in rat plasma (Gallo et al., 2004). However, in this study, this AUC\(_{\text{tumor}}/\text{AUC}_{\text{plasma}}\) ratio determined on day 5 in the CD group and those determined on day 1 and day 28 in the MD group were all slightly greater than unity, which raises the question of whether other mechanisms of drug accumulation besides passive diffusion are involved. An alternative explanation for the high TMZ AUC\(_{\text{tumor}}/\text{AUC}_{\text{plasma}}\) ratio could be the combined effects of enhanced tumor vascular permeability and reduced IFP found in the TMZ treatment groups (Fig. 4B), which would enable convective transport to come into play (Jain, 1987; Seymour, 1992). Convective transport could cause circulating protein-bound and unbound TMZ to extravasate from the compromised tumor vessels, leading to higher concentrations of TMZ in tumor IF compared with those attained by diffusional transport alone (Gallego et al., 2004). In the present study, a moderate increase in the protein expression levels of VEGF in TMZ-treated tumors was observed in the first few weeks after the start of treatment in the CD group. Overexpression of VEGF may lead to the increase in tumor microvessel permeability, and the increased tumor vascular permeability may have contributed to the significant increase in the AUC\(_{\text{tumor}}/\text{AUC}_{\text{plasma}}\) ratio on day 5 compared with those on day 1 in the CD group. In contrast, VEGF expression levels in the MD group were reduced by day 28, which may partially explain the significant decrease in the AUC\(_{\text{tumor}}/\text{AUC}_{\text{plasma}}\) ratio in the MD group on day 28. It appears then that early in the TMZ treatments increased microvessel permeability coupled with convective transport elevated TMZ interstitial fluid concentrations; however, with continued treatment and abatement of the action of VEGF, the tumor vasculature may have been normalized (Jain, 2005), minimizing convective transport and restoring diffusion as the essential transport mechanism.

In conclusion, we have been able to identify several PK/PD factors that contribute to the antitumor activity of the MD TMZ therapy. The MD TMZ regimen applied in this study exhibits effectiveness comparable to that of the CD TMZ regimen in suppressing tumor growth, most likely by its action on tumor cells, and the collateral antiproliferative activity against endothelial cells of the growing vasculature in tumors. The moderate effect of the MD TMZ therapy on VEGF and HIF-1\(\alpha\) expressions in tumors support the possibility that the MD regimen may be superior to the CD regimen by normalizing tumor vasculature and preventing tumors from progressing to a proangiogenic state. In addition, by using tumor microdialysis drug concentration measurements it is possible to identify a “breakthrough” dose associated with minimal extravascular drug accumulation, which could indicate whether effective MD regimens require accu-
mulation in tumor cells or whether endothelial cells alone serve as sufficient targets. Although we have not yet identified a breakthrough MD for TMZ, the current study indicates that TMZ activity as a MD agent requires drug accumulation in tumor cells.

References
Bocci G, Nicolaou KC, and Kerbel RS (2002) Protracted low-dose effects on human tumor cells or whether endothelial cells alone serve as sufficient targets. Although we have not yet identified a breakthrough MD for TMZ, the current study indicates that TMZ activity as a MD agent requires drug accumulation in tumor cells.

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