Pharmacodynamic-Mediated Effects of the Angiogenesis Inhibitor SU5416 on the Tumor Disposition of Temozolomide in Subcutaneous and Intracerebral Glioma Xenograft Models

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ABSTRACT

The objective of this study was to determine the tumor distribution of temozolomide, an alkylating agent, in the absence and presence of the angiogenesis inhibitor 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]indolin-2-one (SU5416), a specific vascular endothelial cell growth factor receptor 2 inhibitor. The study was conducted in nude rats bearing either subcutaneous or intracerebral tumors that overexpressed vascular endothelial cell growth factor. For both tumor locations, animals were assigned to either of two treatment groups, SU5416 (25 mg/kg, dissolved in dimethyl sulfoxide) or vehicle control, dimethyl sulfoxide (710 μl/kg) administered i.p. every day for a total of nine doses. Twenty-four hours after the last dose of SU5416 or dimethyl sulfoxide, temozolomide was administered as a steady-state infusion regimen designed to achieve target plasma concentrations (C∞) of 20 μg/ml. In addition to the measurement of temozolomide C∞, tumor interstitial fluid unbound concentrations of temozolomide were evaluated by microdialysis. In subcutaneous tumors, SU5416 treatment produced a 24% reduction in steady-state temozolomide C∞ values (p < 0.05) as well as 21% reductions in tumor/plasma concentration ratios (C∞/Cp, p = 0.11) compared with controls. In intracerebral tumors, steady-state temozolomide C∞ and C∞/Cp ratios were significantly increased by 2-fold in the SU5416 treatment group compared with control. The apparent paradoxical effect of SU5416 on the tumor disposition of temozolomide in subcutaneous and intracerebral tumors is discussed in the context of physiological changes (for example, interstitial fluid pressure and microvessel density) and the sampling region in the tumor. It is proposed that the net balance of antiangiogenic drug-mediated pharmacodynamic actions will determine how drug disposition in tumors may be affected.

The ability to combine anticancer drugs into effective therapeutic regimens is a cornerstone of successful cancer chemotherapy. A common theme is to combine agents with different mechanisms of action and nonoverlapping toxicities as a means to improve the therapeutic index. The combination of angiogenesis inhibitors and cytotoxic agents represents two classes of drugs that exemplify this latter dogma of combining drugs with different pharmacological targets and different toxicities. Antiangiogenic drugs inhibit endothelial cell proliferation and neovascularization by various mechanisms, with many of the newer agents able to interrupt different growth factor pathways. Cytotoxic drugs target tumor cells and cause cell death through a diverse range of mechanisms, and most often possess a dose-limiting toxicity of myelosuppression. In preclinical models the combination of angiogenesis inhibitors and cytotoxic drugs compared with single agent therapy have shown increased efficacy based on endpoints of tumor growth (Kato et al., 1994; Teicher et al., 1996; Cascinu et al., 1999; Browder et al., 2000). Except for our previous investigations (Devineni et al., 1996; Ma et al., 2001), there have been no pharmacokinetic investigations of combinations of antiangiogenic and cytotoxic drugs.

The growth and metastatic nature of tumors are intimately dependent on the process of angiogenesis. This multistep process may be characterized by the angiogenic phenotype, which includes the breakdown of capillaries and a state of vascular hyperpermeability. We had hypothesized that successful antiangiogenic therapy would reverse the state of vascular hyperpermeability leading to a normal vascular phenotype that would be less permeable to the transport of low molecular weight anticancer drugs. In support of this proposal, we showed that O-(chloroacetyl-carbamoyl) fumagillol (TNP-470), an antiangiogenic compound, caused significant reductions in the tu-
or concentrations of temozolomide, an alkylating agent (Devineni et al., 1996; Ma et al., 2001). The reductions in temozolomide concentrations were found in both subcutaneous and intracerebral gliomas that overexpressed vascular endothelial cell growth factor (VEGF), a key angiogenic factor, but not in isogenic tumors with low VEGF expression (Ma et al., 2001). Because it was found that microvessel density in tumors also decreased in the TNP-470 treatment groups, the reductions in temozolomide concentrations were attributed to TNP-470’s action on vascular density and permeability. In addition, because TNP-470 and temozolomide did not interact pharmacokinetically, the reduced tumor concentrations of temozolomide were attributed to the pharmacodynamic actions of TNP-470 on the tumor vasculature.

The current investigation was designed to extend our evaluation of interactions between angiogenesis inhibitors and cytotoxic drugs by use of another angiogenesis inhibitor, SU5416, a specific receptor tyrosine kinase inhibitor of VEGFR2 located on endothelial cells (Fong et al., 1999; Mendel et al., 2000; Smolich et al., 2001). Evaluation of the SU5416/temozolomide interaction was conducted in nude rats bearing either subcutaneous or intracerebral tumors that overexpressed VEGF (V+) (Ma et al., 1998), and incorporated microdialysis to assess tumor interstitial fluid temozolomide concentrations, the analogous model used in the TNP-470/temozolomide interaction studies (Ma et al., 2001).

Materials and Methods

Materials

Temozolomide and SU5416 were kindly provided by Schering-Plough Research Institute (Kenilworth, NJ) and Sugen Pharmaceutical Company (South San Francisco, CA), respectively. Soft-tissue microdialysis probes (CMA/20), brain microdialysis probes (CMA/12), a refrigerated fraction collector (CMA/170), and a freely moving animal restraint system were purchased from CMA Microdialysis (North Chelmsford, MA). Male adult nude rats (170–240 g b.w.t.) were purchased from Taconic Farms (Germantown, NY). HPLC analyses were performed with a Hewlett Packard model 1050 liquid chromatography system. All other chemicals and supplies were obtained from commercial sources.

Tumor Model and Implantation

Our previously established human glioma cell line SF188/V+ was used throughout the investigations (Ma et al., 1998). This model is based on the parental human SF188 cell line that had been found to have low VEGF expression. A subline, SF188/V+ or V+ that overexpressed VEGF was derived from SF188 cells by transfection with the mouse full-length VEGF164 cDNA as reported previously (Ma et al., 1998). Cells were grown as monolayers in Dulbecco’s Modified Eagle’s medium containing 10% fetal calf serum and were maintained for two consecutive days. The weight loss was normally accompanied by the appearance of central nervous system symptoms (i.e., unsteady gait and arched back) related to the tumor. Vehicle control and SU5416-treated animals received the same volume of dimethyl sulfoxide (710 μl/kg).

Microdialysis, and Temozolomide Dosing

On the last day of SU5416 or vehicle control treatment (day 9), a right common carotid artery and a jugular vein cannula were implanted for temozolomide administration and blood sampling, respectively.

Tumor microdialysis was conducted in both subcutaneous and intracerebral tumors to provide a measure of interstitial fluid unbound or free temozolomide concentrations by the zero-flow microdialysis calibration method (Chaurasia, 1999). On the day of temozolomide administration (1 day after the last dose of SU5416 or vehicle), one microdialysis probe was inserted into the guide cannula and perfused with simulated cerebrospinal fluid (1.1 mM MgCl2, 1.35 mM CaCl2, 0.242 mM NaHPO4·7H2O, 3 mM KCl, 0.242 mM NaHPO4·7H2O, 20 mM NaHCO3, and 131.9 mM NaCl) at 4 μl/min for at least 45 min before the administration of temozolomide. For each intracerebral tumor, a brain microdialysis probe was inserted into the guide cannula and perfused with simulated cerebrospinal fluid (1.1 mM MgCl2, 1.35 mM CaCl2, 3 mM KCl, 0.242 mM NaHPO4·7H2O, 20 mM NaHCO3, and 131.9 mM NaCl) at 4 μl/min for at least 45 min before administration of temozolomide. Temozolomide was then given intra-arterially to achieve steady-state plasma concentrations of 20 μg/ml for at least 7 h by infusing 2 mg/kg/min over 10 min, followed by a 7-h infusion at 0.2 mg/kg/min temozolomide. During the 7-h temozolomide infusions, the microdialysis flow rate was varied from 4, 1, 3, 2, and 6 μl/min, consistent with the zero-flow calibration method (Chaurasia, 1999). At each flow rate from four to six serial dialysate samples were collected in individual vials containing 3.7 to 5.0 μl of 1 N HCl to preserve the chemical stability of temozolomide. The dialysate samples were stored at –80°C until analyzed by HPLC.

The zero-flow microdialysis calibration method is conducted under steady-state temozolomide plasma concentrations in conjunction with variable microdialysis flow rates. It is known that drug recovery across the dialysis membrane and the associated dialysate drug concentrations are a function of the dialysate flow rate, which is used as an independent variable in the exponential equation characterizing the change in temozolomide dialysate concentrations as a func-
tion of flow rate. The equation assumes that other parameters (i.e., dialysis membrane permeability and surface area) are constant during the experiment. The equation is fit to the measured temozolomide dialysate concentrations to obtain an estimate of the zero-flow or actual interstitial fluid concentration. Temozolomide dialysate concentrations collected from the last two fractions (approximately 20- to 30-min periods) at each flow rate were used in the estimation of the zero-flow concentration (Chaurasia, 1999).

**HPLC Analysis of Temozolomide**

Plasma (100 μl) was acidified with 20 μl of 1 N HCl and then 200 μl of cold acetonitrile was added to precipitate proteins. The tubes were vortexed and centrifuged at 15,000 rpm for 5 min. The resulting supernatant (100 μl) was combined with 100 μl of mobile phase that consisted of 5% (v/v) acetonitrile in 0.05 M ammonium acetate buffer, pH 6.8. The mixture was vortexed and centrifuged at 15,000 rpm for 5 min. The resulting supernatant (100 μl) was injected directly onto the HPLC system. Dialysate samples (10 μl) were injected onto the HPLC system, which consisted of a C18 column (150 × 4.6 mm, 5 μm; Alltech Spherisorb, Deerfield, IL), and UV detector. Temozolomide was detected at 232 nm at a flow rate of 0.7 ml/min. An external standard calibration method was used to calculate plasma and dialysate temozolomide concentrations. The HPLC assay was accurate and precise with coefficients of variation of 15% or less.

**Immunohistochemical Staining for CD31**

A standard immunohistochemical assay was used to measure microvessel density (Ma et al., 2001; Pietras et al., 2001). Vascular endothelial cells were stained with an anti-CD31 monoclonal antibody (TLD-3A12; Research Diagnostics, Flanders, NJ) on 5-μm paraffin-embedded sections. Tissue sections incubated without the primary antibody were used as negative controls. After immunostaining, microvessel density in subcutaneous tumors was measured in three different tumor areas, including the tumor/stromal interface. In the intracerebral study, microvessel density was evaluated in at least five different regions. Microvessel density was quantitated by image analysis (Adobe Photoshop) that measured pixel intensity based on a digital selection criteria corresponding to the stained endothelial cells. The percentage of microvessel density in each section was obtained by dividing the intensity of the stained endothelial cells by the total pixel intensity in the same microscopic field.

**Statistical Analyses**

Comparisons between the dimethyl sulfoxide vehicle control and SU5416 treatment groups were made for temozolomide plasma and tumor concentrations, tumor/plasma concentration ratios, and microvessel density using an analysis of variance test (JMP, version 5; SAS Institute, Inc., Cary, NC). Statistical significance was indicated by values of p < 0.05.

**Results**

**Pharmacokinetic Studies.** Two basic types of study designs were available to obtain pharmacokinetic information to evaluate the potential of a drug interaction between SU5416 and temozolomide. In one design, temozolomide concentrations are collected under nonsteady-state conditions, typically after intravascular bolus administrations. The second design uses a temozolomide administration regimen that produces steady-state drug concentrations. Each design enables an assessment of temozolomide administration regimen that produces steady-state drug concentrations. Each design enables an assessment of temozolomide administration regimen that produces steady-state drug concentrations. Under nonsteady-state conditions, tumor uptake can be assessed by the ratio of the area under the drug-concentration time curve in tumor divided by the area under the drug-concentration time curve in plasma, whereas under steady-state conditions the ratio of steady-state tumor to plasma drug concentrations provides an analogous assessment. The current investigation used the steady-state study design because the zero-flow microdialysis calibration method, which is conducted under steady-state conditions, is less prone to calibration errors associated with methods used for nonsteady-state conditions, such as retrodialysis.

**Subcutaneous Tumor Study.** In both the dimethyl sulfoxide vehicle control and SU5416 treatment groups, steady-state temozolomide plasma concentrations were achieved and were 22.2 ± 4.2 and 21.0 ± 3.0 μg/ml, respectively (Fig. 1A). Table 1 shows the physiological and temozolomide pharmacokinetic parameters from the tumor microdialysis study. SU5416 decreased tumor size compared with vehicle control by about 24%. The steady-state tumor interstitial fluid unbound temozolomide concentrations were 16.5 ± 3.7 and 12.6 ± 3.9 μg/ml in control and SU5416 treatment groups, respectively. This 24% reduction in temozolomide tumor concentrations by SU5416 was significant compared with dimethyl sulfoxide control (p = 0.038), yet the difference in the steady-state C0/C0 tumor concentration ratios did not quite reach statistical significance (p = 0.11), being 0.77 ± 0.23 and 0.61 ± 0.23 in control and SU5416 treatment groups, respectively. The C0/C0 concentration ratios reflect differences in the actual steady-state plasma concentrations and provide the most definitive assessment of differences in drug distribution between the two treatment groups. Thus, the reduction in temozolomide’s steady-state distribution into tumors due to SU5416 was not significantly different from the dimethyl sulfoxide control treatment.

**Intracerebral Tumor Study.** Investigation of drug disposition in tumors is readily accomplished when tumors are located subcutaneously because of their defined boundaries and easy access. These features minimize the chance of contamination by normal tissue and ensure that the measured drug concentrations are, in fact, tumoral. Animals tolerate subcutaneous tumors well, and pharmacokinetic studies can be accomplished without significant morbidity or mortality, factors that complicate performing similar studies in animals bearing intracerebral tumors. At the same time, pharmacokinetic studies performed in intracerebral tumor models yield a more mechanistic and clinically relevant characterization of drug disposition because the tumor is located in the normal tissue of origin. Drug transport obstacles presented by the blood-brain barrier and blood-tumor barrier are more likely to resemble the human situation.

Table 2 shows the physiological and temozolomide pharmacokinetic parameters from the intracerebral study. Temozolomide plasma concentrations in the SU5416 and vehicle control groups were similar (p > 0.05), being 19.2 ± 0.5 and 19.3 ± 2.14 μg/ml, respectively (Fig. 1B). The steady-state temozolomide tumor interstitial concentrations were significantly increased (p < 0.05) in the SU5416 treatment group (5.3 ± 2.6 μg/ml) compared with the control group (2.8 ± 1.2 μg/ml). Accordingly, the steady-state temozolomide C0/C0 ratio increased 100% from 0.14 ± 0.05 in the control group to 0.28 ± 0.12 (p < 0.05) in the SU5416 group (Table 2; Fig. 2). This increased temozolomide tumor to plasma concentration ratio is in contrast to that observed in subcutaneous tumors under analogous drug treatment protocols. Possible explanations for the findings are given under Discussion.
Microvessel Density Analysis. A number of biological endpoints are being proposed as pharmacodynamic indices of antiangiogenic therapy (Jain et al., 1997). Among these parameters, microvessel density has been consistently measured based on immunohistochemical methods using different endothelial cell markers. The anti-CD31 method is relatively common and has been shown to be an accurate measure of neovascularization (Giatromanolaki et al., 1997). In subcutaneous tumors, three different regions were used for the analysis; two peripheral regions of the tumor and the stroma, defined as a 100-μm-wide area at the leading edge of the tumor. In the intracerebral group, microvessel density was quantitated in at least five different sites in the tumor. In most cases, the highest microvessel density was within 100 μm of the leading edge of the tumor. There were significant reductions in microvessel density in subcutaneous tumors in the stroma region in SU5416-treated animals compared with control (p < 0.05). There were also reductions in microvessel density in the peripheral regions of subcutaneous tumors (control mean = 37.44 ± 12.74%; SU5416 mean = 28.33 ± 10.98%; p > 0.05); however, this reduction due to SU5416 treatment did not reach statistical significance. Intracerebral tumors exhibited about a 16% reduction of tumor microvessel density (control mean = 8.4 ± 3.0%; SU5416 mean = 7.1 ± 1.8%; p > 0.05) in the SU5416-treated group compared with the control when averaged over all sampled regions. Table 3 provides the microvessel density [mean ± S.D.] measurements for each region and treatment group.

Tumor necrosis could readily be evaluated in subcutaneous tumors because they were easily accessible. Intracerebral tumors were hard to collect as discrete samples because they were smaller, interdigitated with normal brain, and analyzed for microdialysis probe placement. For subcutaneous tumors, 62% of tumors showed greater than 50% necrosis in the dimethyl sulfoxide control, whereas in the SU5416 treatment group only 13% of the tumors showed greater than 50% necrosis.

Discussion

Combination chemotherapy is an important strategy in the treatment of cancer. The ability to combine drugs with different mechanisms of action and nonoverlapping toxicities may enhance efficacy and attenuate the development of drug resistance. In many instances, the design of combination dosing regimens is arrived at semiempirically without detailed pharmacokinetic and pharmacodynamic analyses. Rarely are measurements of drug concentrations in tumor used as a basis to evaluate combination anticancer drug therapy. In consideration of the pharmacodynamic basis of angiogenesis inhibitor and cytotoxic drug interactions, we have emphasized the importance of determining drug concentration measurements in tumors. Our previous work in this area has shown that the angiogenesis inhibitor TNP-470 reduced tumor concentrations of temozolomide in both syngenic and xenograft glioma models (Devnineni et al., 1996; Ma et al., 2001), which were attributed to a reduction in microvessel density and capillary permeability caused by TNP-470. In light of the current study, which found increased temozolomide brain-tumor concentrations after SU5416 treatment, a reanalysis of the mechanisms responsible for interactions between angiogenesis inhibitors and cytotoxic drugs was required.

In all of our investigations, we have used microdialysis to obtain a measure of unbound temozolomide concentrations in the interstitial fluid of tumors. Microdialysis samples collected from subcutaneous V7 tumors were from the tumor periphery, the area most indicative of antiangiogenic activity.
Physiological and temozolomide pharmacokinetic parameters from the subcutaneous tumor microdialysis study

All values represent mean ± SD, n = 7.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Tumor Size Pretreatment</th>
<th>Tumor Size Post-treatment</th>
<th>ss $C_p$ (g/ml)</th>
<th>ss $C_t$ (g/ml)</th>
<th>ss $C_t/C_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide vehicle</td>
<td>1386 ± 631$^a$</td>
<td>1684 ± 873$^b$</td>
<td>22.2 ± 4.2$^a$</td>
<td>16.5 ± 3.7$^c$</td>
<td>0.77 ± 0.23$^b$</td>
</tr>
<tr>
<td>SU5416 (25 mg/kg)</td>
<td>1345 ± 591</td>
<td>1315 ± 546</td>
<td>21.0 ± 2.2$^d$</td>
<td>12.6 ± 3.9$^d$</td>
<td>0.61 ± 0.23$^d$</td>
</tr>
</tbody>
</table>

$^a$ ss $C_p$, steady-state plasma concentrations; $^b$ ss $C_t$, steady-state interstitial fluid tumor concentrations.

Table 2

Physiological and temozolomide pharmacokinetic parameters from the intracerebral tumor microdialysis study

All values represent mean ± SD, n = 6.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Parameters$^a$</th>
<th>$ss C_p$ (g/ml)</th>
<th>$ss C_t$ (g/ml)</th>
<th>$ss C_t/C_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide vehicle</td>
<td></td>
<td>19.3 ± 2.1$^a$</td>
<td>2.8 ± 1.2$^a$</td>
<td>0.14 ± 0.05$^a$</td>
</tr>
<tr>
<td>SU5416 (25 mg/kg)</td>
<td></td>
<td>19.2 ± 4.1$^a$</td>
<td>5.3 ± 2.6$^a$</td>
<td>0.28 ± 0.12$^a$</td>
</tr>
</tbody>
</table>

$^a$ ss $C_p$, steady-state plasma concentrations; $^b$ ss $C_t$, steady-state interstitial fluid tumor concentrations.

In the TNP-470/temozolomide studies, regardless of the tumor location or microdialysis sampling site, TNP-470 treatment always decreased temozolomide tumor concentrations. The reductions in the steady-state $C_t/C_p$, temozolomide concentration ratios were 30 and 50% in the subcutaneous and intracerebral V+ tumors, respectively (Ma et al., 2001). These reductions were associated with nearly analogous reductions of 30 and 63% in microvessel density in the subcutaneous and intracerebral V+ tumors, respectively (Ma et al., 2001), and supported our previous assertion that antiangiogenic therapy decreased cytotoxic drug concentrations by inhibition of capillary density and permeability.

In the SU5416/temozolomide studies, a differential effect of SU5416 was observed on tumor concentrations of temozolomide. In subcutaneous V+ tumors, the action of SU5416 caused a 21% reduction in steady-state tumor/plasma temozolomide concentration ratios. Even though this reduction...
Various angiogenesis inhibitors may have contributed to tumor necrosis, the normal progression of tumor growth and metastasis does lead to hypoxic and necrotic tumors. Thus, the positive action of SU5416 on temozolomide's brain-tumor concentrations may also occur in tumors containing hypoxic regions as a result of common growth patterns of solid tumors.

In conclusion, we have demonstrated that the angiogenesis inhibitor SU5416 resulted in either increased (brain-tumor) or decreased (subcutaneous) temozolomide tumor concentrations. It is believed that this differential result is due to the microdialysis sampling site, central versus peripheral, that may have been enhanced by the ability of dimethyl sulfoxide to cause tumor necrosis. It is proposed that angiogenesis inhibitors can alter multiple tumor physiological variables (i.e., microvessel density, permeability, hypoxia, and interstitial fluid pressure) that can result in pharmacodynamic-mediated changes in tumor drug concentrations. The ultimate effect on cytotoxic drug concentrations will be determined by the net balance of physiological effects that depend on the tumor and the local environment in which drug concentrations are measured. The continued evaluation of angiogenesis inhibitor/cytotoxic drug interactions should incorporate independent assessments of the multiple physiological variables to fully understand the mechanisms underlying changes in drug concentrations in tumors.

### References


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