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Pharmacodynamic-mediated Reduction of Temozolomide Tumor Concentrations by the Angiogenesis Inhibitor TNP-470[1]

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ABSTRACT

The angiogenic phenotype is associated with increased tumor neovascularization and a state of vascular hyperpermeability to macromolecules. Angiogenesis inhibitors could reverse these processes, resulting in tumor capillaries that have normal membrane permeability. It was proposed that the switch from a hyperpermeable to a normal permeable state could have the untoward effect of decreasing tumor concentrations of anticancer drugs coadministered with angiogenesis inhibitors. The current investigation evaluated a potential drug interaction between the angiogenesis inhibitor *O*-(*N*-chloroacetyl-carbamoyl)-fumagillol (TNP-470) and the alkylating agent temozolomide (TMZ), in xenograft models that differentially expressed vascular endothelial growth factor (VEGF), a driving force for angiogenesis. Nude rats bearing either s.c. low VEGF (V−) or high VEGF (V+) or intracerebral V+ gliomas were administered either a multiple-dose regimen of TNP-470 or vehicle control. One day after the last dose of vehicle or TNP-470, a steady-state dosing regimen of TMZ was administered with subsequent collection and high-performance liquid chromatography analysis of plasma and either tumor homogenate or tumor microdialysis steady-state TMZ concentrations, and in some cases [5-(3-methyltriazen-1-yl)imidazole-4-carboximide] MTIC, its active metabolite. Microvessel density (MVD) was quantitated by image analysis using an anti-CD31 method. Statistical analyses of pharmacokinetic and pharmacodynamic end points in the control and TNP-470 treatment groups were completed by nonparametric tests. In both the s.c. and intracerebral V+ models, TNP-470 treatment produced significant reductions in TMZ tumor concentrations and tumor:plasma concentration ratios compared with control, being reduced an average of 25% and 50% in the s.c. and intracerebral tumors, respectively. MTIC concentrations in V+ s.c. tumors also were reduced by 50% in the presence of TNP-470. Consistent with the lower extent of neovascularization in the V− tumors, TMZ and MTIC tumor concentrations were not different in TNP-470 and control treatment groups in s.c. tumors. MVD was reduced by TNP-470 compared with vehicle control in the V+ tumors, but was unaltered in V− tumors, attesting to the use of MVD as a pharmacodynamic end point and the effectiveness of TNP-470 as an angiogenesis inhibitor. Angiogenesis inhibitor's pharmacodynamic actions on tumor angiogenesis can produce a reduction in tumor concentrations of coadministered anticancer agents. It is increasingly important to understand the pharmacokinetic and pharmacodynamic behavior of each class of drug so that optimal dosing regimens can be designed.

INTRODUCTION

Angiogenesis is considered an integral process to the growth and spread of solid tumors and has become a growing focus of anticancer therapy. Endothelial cells, the primary target of antiangiogenic therapy, may offer advantages over tumor cell targets, such as genetic stability and greater physiological accessibility (1). A variety of angiogenesis inhibitors have demonstrated antitumor activity both as single agents and in combination with tumor cell-targeted cytotoxic agents in a host of preclinical models (2–4). Results from such efficacy studies, and the distinct cell targets of angiogenesis inhibitors and cytotoxic drugs, support a therapeutic strategy of combined antiangiogenic and cytotoxic drug chemotherapy.

It is known that tumor cells overexpress angiogenic factors such as basic fibroblast growth factor and VEGF[3] that bind to their cognate receptors on the surface of endothelial cells. These growth factors stimulate neovascularization, or the growth of new capillaries that supply tumor cells with physiological components necessary to maintain a positive growth balance. Tumor neovascularization is an integral component of the angiogenic switch that is associated with a state of vascular hyperpermeability (5). The hyperpermeable state permits plasma proteins or macromolecules to traverse a normally impermeable capillary endothelial cell barrier, creating a new provisional extracellular matrix that ultimately leads to new vessel formation (6, 7). The enhanced ability of macromolecules to extravasate has been attributed to both transendothelial and interendothelial cell pathways that include the involvement of fenestrae, cytokines, and vesicular-vacuolar organelles that function to shuttle macromolecules through capillary endothelial cells. Angiogenesis inhibitors work through different mechanisms of action, yet invariably their utility will be based on their ability to inhibit angiogenesis. This action could revert the hyperpermeable state to a normapermeable state, thus terminating protein extravasation.

The effects of a hyperpermeable tumor vasculature and the associated consequences of angiogenesis inhibitors on drug transport is largely unknown. Previously we have shown that TNP-470 reduced interstitial fluid concentrations of TMZ in a rat syngeneic model (8). TNP-470 is a semisynthetic analogue of fumagillin that inhibits endothelial cell growth by initial binding to type 2 methionine aminopeptidase (9). TNP-470 is being evaluated in a number of clinical trials (10).[4] TMZ is a second-generation imidazotetrazine prodrug of low molecular weight that is used in the treatment of malignant gliomas and other difficult tumors (11). It undergoes spontaneous conversion at physiological pH to MTIC, the active DNA alkylating species. Our previous investigation (8) of a TNP-470:TMZ drug interaction measured TMZ interstitial fluid concentrations in the central region of s.c. rat C6 tumors. TMZ concentrations were assessed using tumor microdialysis with a retrograde calibration method. The current investigation was designed to extend this work by the use of xenograft models that differentially expressed VEGF (12) and to evaluate the potential drug interaction in both intracerebral gliomas as well as s.c. gliomas. The intracerebral tumor site would also provide an assessment of the role of the BBB on TMZ tumor uptake and serve as a more relevant model for human brain tumors. In addition, the current investigation used two different techniques to assess TMZ concentrations in tumors. In one, a tumor homogenate sample permitted both central and peripheral regions of the tumor to be analyzed; and in the other, a zero-flow microdialysis technique eliminated the need for a retrograde calibration marker (13).

The fundamental premise of the investigation was that an angio-

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; TMZ, temozolomide; TNP-740, *O*-(*N*-chloroacetyl-carbamoyl)-fumagillol; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboximide; BBB, blood-brain barrier; HPLC, high-performance liquid chromatography; ACN, acetonitrile; MVD, microvessel density.

[4] See also Internet address http://cancertrials.nci.nih.gov/news/angio/table.html.

genesis inhibitor would revert hyperpermeable capillaries to a normal permeable state in tumors that overexpressed VEGF, our V+ model, causing a concordant reduction in cytotoxic drug concentrations in tumors. Parallel studies with tumors that did not overexpress VEGF, or V− tumors, were predicted to yield tumor drug concentrations that were unaffected by angiogenesis inhibitors.

MATERIALS AND METHODS

Materials

The human glioma cell line SF188 was kindly provided by the Brain Tumor Research Center, University of California, San Francisco, CA. TMZ and its metabolite, MTIC, were kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). TNP-470 was generously supplied by TAP Pharmaceuticals (Deerfield, IL). Soft-tissue microdialysis probes (CMA/20), brain microdialysis probes (CMA/12), a refrigerated fraction collector (CMA/170), and the Freely Moving Animal Restraint System were purchased from CMA Microdialysis (North Chelmsford, MA). Male nude rats (rnu/rnu) were purchased from Taconic Farms, Inc. (Germantown, NY). HPLC analyses were performed with a Hewlett Packard Model 1050 liquid chromatographic system (Sunnyvale, CA). All other chemicals and supplies were obtained from commercial sources.

Subcutaneous Glioma Model

All animal protocols were approved by the IUPAC in accordance with NIH guidelines. Our previously established human glioma xenograft model was used throughout the investigations (12). This model is based on the parental human SF188 glioma cell line, which had been found to have low VEGF expression, and henceforth referred to as V− cells. A cell line that overexpressed VEGF was derived from V− cells by transfecting with the mouse full-length $VEGF_{164}$ cDNA, as reported previously (12) and referred to as V+ cells. All cell lines were grown as monolayers in DMEM containing 10% FCS and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Male nude rats, ~7–8 weeks of age, were briefly anesthetized with ether and had implanted approximately 5×10^6 V+ and 10×10^6 V− cells, which had been previously suspended in 0.4 ml of Matrigel (Collaborative Biomedical, Bedford, MA), s.c. in the dorsal neck region. After cell implantation, animals were returned to their cage and fed standard rat diet and water *ad libitum*. Animal body weight and tumor size were measured regularly throughout the study periods. Tumor volume was calculated as: tumor volume (cm^3) = $0.5ab$ (2), where a is the longest diameter and b is the shortest diameter. The relative change in tumor volume during the treatment courses was calculated as: 100 × (tumor volume following the last dose of TNP-470 or control − tumor volume prior to the first dose of TNP-470 or control)/(tumor volume prior to the first dose of TNP-470 or control).

Tumor Homogenate Study—s.c. Site

When tumors had grown to ~12 mm in the longest dimension, either 30 mg/kg or 60 mg/kg of TNP-470 were administered to the animals s.c. every 48 h for a total of five doses. This schedule of TNP-470 is similar to regimens used previously which were demonstrated to possess antiangiogenic activity in preclinical models (14, 15). Control animals were treated the same as the TNP-470 groups except that they received vehicle alone, which consisted of 1% ethanol and 5% gum arabic in normal saline. On the last day of TNP-470 therapy, a right common carotid artery and a jugular vein cannula were surgically implanted for drug administration and blood sampling, respectively.

The day after the last dose of TNP-470 or vehicle, TMZ (dissolved in 25% DMSO) was given intraarterially to achieve steady-state plasma concentrations of 40 μg/ml for 2 h by infusing 4 mg/kg/min of TMZ over 10 min, followed by a 110-min infusion of TMZ at a rate of 0.4 mg/kg/min. Plasma samples (400 μl) were collected in chilled, heparinized syringes at 15, 30, 60, and 120 min during the TMZ administrations. The heparinized blood was centrifuged at 4°C and 200 μl of plasma was immediately transferred to a chilled vial and frozen at −80°C until HPLC analysis. After 2 h, the animals were euthanized and samples were collected at the central and peripheral regions of the tumor. Tumor samples were immediately frozen and stored at −80°C until HPLC analysis.

Tumor Microdialysis Study—s.c. Site

A second s.c. tumor model study was conducted that used microdialysis to measure unbound TMZ concentration in interstial fluid. All tumor cell implantations, surgical protocols, and the TNP-470 treatment schedule (only the 30 mg/kg TNP-470 regimen was used) were the same as in the tumor homogenate study.

On the day of TMZ administration (1 day after the last dose of TNP-470 or vehicle), microdialysis probes were inserted into the peripheral region of the tumor and perfused with Ringer's solution at 4 μl/min for at least 45 min before the TMZ administrations. TMZ was then given intraarterially to achieve steady-state plasma concentrations of 20 μg/ml for at least 7 h by infusing 2 mg/kg/min of TMZ over 10 min with a subsequent 7-h infusion of TMZ at a rate of 0.2 mg/kg/min. During the 7-h TMZ infusion, the microdialysis flow rate was varied from 4, 1, 3, 2, and 6 μl/min consistent with the zero-flow calibration method (13). At each flow rate, from four to six serial dialysate samples were collected in individual vials containing 5 μl of 1 N HCl to preserve the chemical stability of TMZ. The dialysate samples were stored at −80°C until analyzed for TMZ by HPLC, as described below. Multiple plasma samples (~12/animal) were collected during the 7-h TMZ infusion and then stored at −80°C until analyzed for TMZ by HPLC.

The zero-flow microdialysis calibration method is conducted under steady-state TMZ 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. A standard formula is fit to the measured TMZ dialysate concentrations from the last two dialysate fractions at each flow rate to provide an estimate of the zero-flow TMZ concentration, which is the actual interstitial fluid concentration (13).

Intracerebral Glioma Model and Tumor Microdialysis Study

The analogous V+ glioma model used for the s.c. model was used for the intracerebral model. The V− model was not studied at an intracerebral site on the basis of the results from the above V− s.c. model. Rats were anesthetized with an i.p. dose (0.1 ml/100 g body weight) of a 3:2:1 (v/v/v) mixture of ketamine hydrochloride (100 mg/ml), acepromazine maleate (10 mg/ml), and xylazine hydrochloride (20 mg/ml) and secured in a stereotaxic apparatus. The scalp was cleaned with 70% alcohol and the skull exposed by a midline incision. Tissue covering the skull was gently everted and the bregma identified. A small burr hole was drilled at a position 2 mm posterior, and 2 mm lateral from the bregma above the thalamic region. Two additional partial small holes were drilled for bone anchor screws several millimeters away from the burr hole. After insertion of the bone anchor screws, 2 μl of the V+ cell suspension (10^8 cells/ml) was slowly injected over 1 min into the right thalamic region at depth of 4.5 mm. After a waiting period of 1 min, the needle was slowly removed from the brain, and a guide cannula was slowly lowered to a depth of 3 mm. Dental cement was used to anchor the guide cannula and the supporting screws. The incision was sutured to cover the cement, leaving only the cannula exposed. The animals were then returned to the cages and received the regular rat diet and water *ad libitum*. Animal body weight was measured frequently throughout the study period.

The intracerebral study protocol was analogous to the s.c. site microdialysis study in terms of the TNP-470 dose regimen, TMZ steady-state dosing regimens, pharmacokinetic sampling, and the zero-flow microdialysis calibration method with two exceptions: (*a*) animals received between four and six TNP-470 30 mg/kg doses every other day, rather than the preset five doses in the s.c. investigations. The variable number of TNP-470 doses coincided with the presentation of the central nervous system symptom complex of unsteady gait, arched back, and unkempt appearance deemed required prior to TMZ administration. Administration of TMZ under such conditions would typically ensure tumors were of sufficient size for pharmacokinetic sampling. Because the size of the intracerebral tumor could not be monitored, the treatment course of either TNP-470 or vehicle control was initiated once animals lost body weight on two consecutive days, typically about 5 g/day. This symptom signified that the effects of the tumor would progress to the central nervous system complex mentioned above over a four- to six-dose course of TNP-470 or vehicle control; and (*b*) the intracerebral microdialysis probes were perfused with simulated cerebrospinal fluid (1.1 mM $MgCl_2$, 1.35 mM $CaCl_2.H_2O$, 3 mM KCl, 0.242 mM $Na_2HPO4.7H_2O$, 20 mM $NaHCO_3$, and 131.9 mM NaCl) rather

than Ringer's solution. On the last day of TNP-470 therapy, right common carotid artery and jugular vein cannulas were surgically implanted for TMZ administration and blood sampling, respectively. All plasma and dialysate samples were stored at −80°C until analyzed for TMZ by HPLC as described below.

Drug Analyses

Two separate analytical methods were used based on previous investigations (8, 16). The tumor homogenate study required methods to quantitate both TMZ and MTIC, whereas in the microdialysis studies, only TMZ was measured.

Tumor Homogenate TMZ and MTIC Analyses. In an ice bath, preparing only six samples at a time, plasma samples were spiked with 100 μl of hydrochlorothiazide in methanol as an internal standard. Samples were vortexed and centrifuged at 4°C for 2 min. The supernatant was immediately transferred into HPLC insert vials and stored at −80°C. One sample was thawed at a time, and 20 μl were injected onto the HPLC system consisting of a Synchropak SCD 100 column (150 mm × 4.6 mm) and a mobile phase of 8% ACN in 0.02 M ammonium acetate that was pumped at a flow rate of 1.1 ml/min. Tumor samples were homogenized on ice with cold methanol (0.4 g/ml). In an ice bath, the tumor homogenate (500 μl) was then spiked with 50 μl of the internal standard. Samples were then vortexed and centrifuged at 15,000 rpm for 1 min. Immediately after centrifuging, the supernatant (200 μl) was put into the HPLC insert vials and placed at −80°C. One sample at a time was thawed, and 20 μl of the sample were injected onto the HPLC system described above. MTIC and TMZ were detected at 316 nm and converted to plasma or tumor concentrations using an internal standards method.

TMZ Analyses from Microdialysis Studies. The acidified plasma samples were protein-precipitated with 200 μl of cold ACN. 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) ACN in 0.05 M ammonium acetate buffer (pH 6.8). The mixture was vortexed and 10 μl of the sample injected onto the HPLC system. Dialysate samples (10 μl) were injected directly onto the HPLC system, which consisted of an Alltech Sperisporb CN (150 mm × 4.6 mm; 5 μm) column. TMZ was detected at 323 nm at a flow rate of 0.7 ml/min and converted to plasma and dialysate concentrations using an external standards method. Interstitial fluid TMZ concentrations were obtained from TMZ dialysate concentrations based on the steady-state zero-flow method (13).

MVD

A standard immunochemical assay procedure was used to stain vascular endothelial cells using a primary monoclonal anti-CD31 antibody (TLD-3A12; Research Diagnostics, Inc., Franders NJ) on 5-μm paraffin-embedded sections. Tissue sections were incubated without primary antibody as a negative control. After immunostaining, MVD was quantitated in three different areas in s.c. tumors, two peripheral regions (an inner region, and one at the leading edge of the tumor) and in stroma within 100 μm of the tumor:stromal interface. On the basis of the smaller size of intracerebral tumors, a single measure of MVD was obtained from areas that showed the strongest staining. MVD measurements were available from four of six animals in the V+ TNP-470 group because of sample handling problems related to the determination of the location of the microdialysis probe. For each area, four to five regions of interests were used for quantitation by image analysis (Adobe Photoshop) that measured pixel intensity based on a digital-selection criteria corresponding to the stained endothelial cells. The percentage of MVD 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

The nonparametric Wilcoxon test (17) was used to determine statistical significance of the study parameters (*i.e.,* drug concentrations, drug concentration ratios, and MVD) between the treatment groups. A $P < 0.05$ was considered significant.

RESULTS

TMZ and MTIC Concentrations from s.c. Tumor Homogenate Study. Analysis of total TMZ and MTIC tumor concentrations by whole-tumor sampling was used in conjunction with tumor microdialysis to assess the nature of a TNP-470:TMZ interaction. The use of whole tumors and the associated tumor homogenates permitted the simultaneous measurement of both TMZ and MTIC in the same tumor sample as well as clear demarcation of central and peripheral tumor regions. The pH-dependent chemical stability of both TMZ (*i.e.,* acid-stable) and MTIC (*i.e.,* base-stable) prevented the simultaneous collection of microdialysis samples in a single medium that would preserve the chemical stability of each drug. Although it is possible to place two microdialysis probes in a single tumor, permitting collection of dialysate samples from a central and peripheral region, we found it to be technically burdensome and unreproducible. The microdialysis technique provided a measure of unbound or free drug concentration that would complement the total drug concentration measurements obtained from the tumor homogenates. Thus, not only could new information be gained from the analysis of total drug concentrations via whole-tumor sampling, it would also serve to support data obtained by tumor microdialysis.

The TNP-470 treatment regimens, at both the 30 mg/kg and 60 mg/kg dose levels, decreased the growth rate of both V− and V+ tumors compared with the vehicle control groups (Table 1), as expected. There was a faster growth rate in the V+ control group (*i.e.,* 128%) compared with the V− control group (*i.e.,* 46.9%), consistent with the effect of VEGF on neovascularization. There was a dose-dependent reduction in V+ tumor volumes attributable to TNP-470. In comparing the relative changes in tumor volume between control and the 30 mg/kg and 60 mg/kg TNP-470 treatment groups, there were V+ tumor volume reductions of 84% (128−44%; Table 1), and 148% [128−(−20%)], respectively. TNP-470 therapy also produced relative reductions in tumor volumes in the V− tumors (Table 1), but it was not as pronounced an effect as in the V+ tumors. This can be seen by comparing the relative tumor volumes in the control group and the 30 mg/kg and 60 mg/kg TNP-470 treatment groups that yield reductions in tumor size of 61.9% [46.9−(−15%)[and 65.9% [46.9−(−19%)], respectively. The smaller TNP-470 effect in the V- tumors could be attributed to the lower state of neovascularization in the V− tumors and the likelihood that near-maximal growth retardation was achieved at the lower 30 mg/kg dose level. Body weight decreased from a mean range of 3–13% during the TNP-470 treatment courses compared with increases from 6 to 13% in the vehicle control groups, consistent with reported TNP-470-induced weight loss (18). In summary, the tumor volume parameters indicated TNP-470 was pharmacologically active based on its effects on tumor growth in V+ and V− tumors.

Three questions were addressed in the s.c. tumor homogenate investigation based on total TMZ and MTIC concentrations and their

Table 1 *Relative changes in subcutaneous tumor volumes during the course of vehicle and TNP-470 treatment*

	Tumor type	
Treatment[a]	V−	V+
Vehicle control	46.9 ± 76%[b]	128 ± 79%
TNP-470 30 mg/kg	−15 ± 28%	44 ± 32%
TNP-470 60 mg/kg	−19 ± 20%	−20 ± 30%

[a] All treatments consisted of five doses administered every 48 h.

[b] All values are mean ± SD, with $n = 4$ except the TNP-470 60 mg/kg group, in which $n = 3$. The relative change in tumor volume during the treatment courses was calculated as: 100 × (tumor volume following the last dose of TNP-470 or control − tumor volume prior to the first dose of TNP-470 or control)/(tumor volume prior to the first dose of TNP-470 or control).

Table 2 *Physiological and pharmacokinetic parameters from the subcutaneous tumor homogenate study*

Parameter[a]	V− treatment groups[b]			V+ treatment groups		
	Control	TNP 30 mg/kg	TNP 60 mg/kg	Control	TNP 30 mg/kg	TNP 60 mg/kg
Tumor size [mm^3]	1878 ± 626	1374 ± 1033	1755 ± 1779	2389 ± 659	2023 ± 478	1064 ± 349
Body weight (g)	275 ± 50	282 ± 41	302 ± 7	215 ± 37	214 ± 42	237 ± 19
ss C_p TMZ (μg/ml)	48.0 ± 6.0	54 ± 5.8	58.7 ± 21.4	48.0 ± 6.7	54.3 ± 3.9	41.0 ± 4.0
ss C_t TMZ[c] (μg/ml)	18.8 ± 6.6	15.1 ± 3.2	16.5 ± 3.6	39.5 ± 8.4	27.5 ± 6.2[d]	19.5 ± 6.7[d]
ss C_t/C_p TMZ	0.39 ± 0.09	0.28 ± 0.03	0.30 ± 0.09	0.83 ± 0.15	0.51 ± 0.10[d]	0.47 ± 0.11[d]
ss C_p MTIC (μg/ml)	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	1.3 ± 0.3	1.0 ± 0.2
ss C_t MTIC (μg/ml)	0.49 ± 0.08	0.47 ± 0.03	0.52 ± 0.02	0.97 ± 0.20	0.45 ± 0.05[d]	0.54 ± 0.10[d]
ss C_t/C_p MTIC	0.50 ± 0.07	0.44 ± 0.09	0.57 ± 0.26	1.02 ± 0.16	0.38 ± 0.10[d]	0.53 ± 0.07[d]

[a] ss C_p, steady-state plasma concentration; ss C_t, steady-state whole tumor concentrations. Body weights and tumor volumes are reported from the last day of vehicle or TNP-470.
[b] All values are mean ± SD with $n = 4$ in all groups except the TNP-470 60 mg/kg group, in which $n = 3$.
[c] All TMZ and MTIC tumor concentrations reported from the peripheral region of the tumor.
[d] Significantly different than corresponding control group; $P < 0.05$.

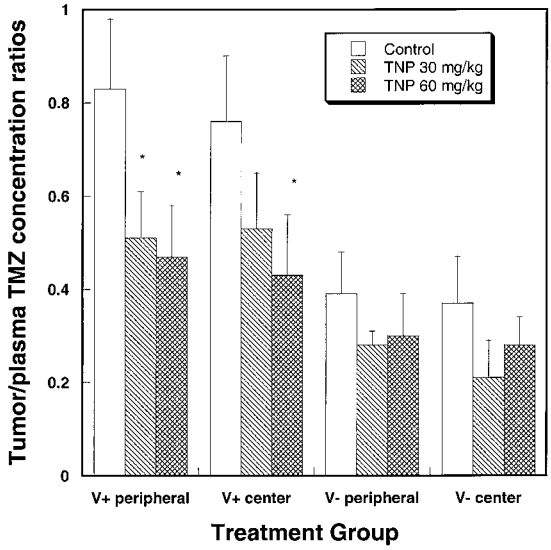

Fig. 1. Steady-state TMZ tumor:plasma concentration ratios. Mean (+ 1 SD) ratios were obtained in vehicle control-, TNP-470 30 mg/kg-, and TNP-470 60 mg/kg-treated nude rats bearing s.c. human tumors obtained from either parental SF188 glioma cells (*V*−) or overexpressing VEGF SF188 glioma cells (*V*+). Tumors were collected 1 day after the last dose of a five-dose cycle of either TNP-470 or vehicle after administration of a 2-h steady-state infusion regimen of TMZ. ∗, significantly different from control at the $P < 0.05$ level; $n = 3$ or 4 animals/group.

tumor:plasma ratios: (*a*) did TNP-470 alter tumor drug concentrations compared with control? (*b*) were there drug concentration differences between central and peripheral tumor regions? and (*c*) were drug concentrations different between V− and V+ tumors? The 2-h steady-state regimen of TMZ was used to ensure and document that steady-state conditions were achieved by measurement of plasma TMZ concentrations throughout the 2-h infusion. Steady-state TMZ plasma concentrations were achieved in all treatment groups and ranged from 41.4 ± 4 μg/ml to 58.7 ± 21.4 μg/ml (Table 2). Analysis of tumor homogenate TMZ concentrations revealed a number of differences in steady-state tumor:plasma concentration ratios between the treatment groups as shown in Fig. 1. In the V+ tumors, both the 30 mg/kg and 60 mg/kg TNP-470 dose regimens significantly decreased TMZ tumor concentrations and concentration ratios by ~25% compared with vehicle control at the tumor periphery. Similar reductions were observed at the center of the tumor; however, the reductions in TMZ concentration ratios were only significant at the higher TNP-470 dose level (Fig. 1). In contrast with the results in the V+ treatment groups, there were minimal alterations in TMZ tumor concentrations and concentration ratios in the V− groups at both the tumor periphery and center, regardless of the TNP-470 dose level (Fig. 1). These data clearly support the ability of TNP-470 to mediate a reduction in TMZ tumor concentrations in V+ tumors, but not in V− tumors, consistent with the role of VEGF in neovascularization and membrane permeability.

Additional analysis of the TMZ tumor:plasma concentration ratios in Fig. 1 indicate that there were not significant differences between central and peripheral regions for analogous treatments (*i.e.*, compare vehicle control or TNP-470 groups) in either V+ or V− tumors. This may be attributed to a more uniform vasculature throughout the tumor and the lack of central necrotic areas because of the relatively small tumor sizes. Comparison of TMZ tumor:plasma concentration ratios revealed almost 2-fold higher values in the V+ groups compared with the analogous V− groups. This is indicative of the greater vascularity and membrane permeability in the V+ tumors compared with the V− tumor, even in the presence of TNP-470 therapy.

Examination of MTIC, the active metabolite of TMZ, tumor concentrations, and tumor:plasma concentration ratios (Fig. 2) revealed a similar pattern of changes among the treatment groups, as observed for TMZ. Again as with TMZ, steady-state MTIC plasma concentrations were obtained in all treatment groups (Table 2) and were not significantly different. Both the 30 mg/kg and 60 mg/kg TNP-470 treatment regimens caused an ~50% reduction in MTIC tumor concentrations and concentration ratios compared with vehicle control (Fig. 2). These reductions occurred at both the tumor center and

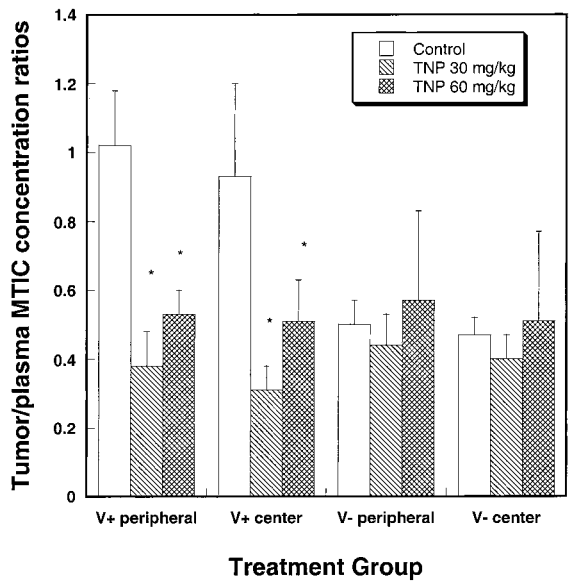

Fig. 2. Steady-state MTIC tumor:plasma concentration ratios. Mean (+ 1 SD) ratios were obtained in vehicle control-, TNP-470 30 mg/kg-, and TNP-470 60 mg/kg-treated nude rats bearing s.c. human tumors obtained from either parental SF188 glioma cells (*V*−) or overexpressing VEGF SF188 glioma cells (*V*+). Tumors were collected 1 day after the last dose of a five-dose cycle of either TNP-470 or vehicle after administration of a 2-h steady-state infusion regimen of TMZ. ∗, significantly different from control at the $P < 0.05$ level; $n = 3$ or 4 animals/group.

Table 3 *Physiological and TMZ pharmacokinetic parameters from the subcutaneous tumor microdialysis study*

Parameter[a]	Treatment group			
	V− Control	V− TNP-470	V+ Control	V+ TNP-470
Tumor Size (mm^3)	1220 ± 760[b]	910 ± 627	2286 ± 1160	1081 ± 506
Body weight (g)	335 ± 40	315 ± 56	309 ± 35	271 ± 52
ss C_p (μg/ml)[c]	25.6 ± 4.5	27.7 ± 7.2	22.5 ± 1.5	23.9 ± 7.2
ss C_{tif} (μg/ml)	12.3 ± 2.9	12.9 ± 2.7	16.6 ± 4.3	12.0 ± 1.8
ss C_{tif}/C_p	0.49 ± 0.11	0.49 ± 0.17	0.73 ± 0.15[d]	0.52 ± 0.09

[a] ss C_p, steady-state TMZ plasma concentration; ss C_{tif}, steady-state tumor interstitial fluid TMZ concentrations. Body weights and tumor volumes are reported from the last day of vehicle or TNP-470.
[b] Values reported as mean ± SD; n = five or six animals/group.
[c] ss C_p values are averaged over time and animals. Each animal had ~12 timed samples.
[d] Significantly different than V+ TNP-470 group; $P < 0.05$.

periphery. In the V− group, MTIC tumor concentrations and concentration ratios were relatively uniform among all treatment groups, with no significant differences. Consistent with TMZ tumor concentrations, MTIC concentrations were 2-fold higher in the V+ groups compared with the analogous V− groups. Therefore, based on the MTIC data, one arrives at the same conclusions as with the TMZ data regarding the inhibitory effect of TNP-470 on tumor concentrations, the lack of drug concentration differences in central and peripheral regions, and the greater extent of drug uptake in V+ *versus* V− tumors. In addition, the active alkylating species MTIC does not have a compensatory mechanism, as anticipated for a first-order metabolic conversion, to the reduced TMZ tumor concentrations, and it is plausible that the reduced MTIC tumor concentrations would yield suboptimal effects on tumor growth.

TMZ Interstitial Fluid Concentrations from s.c. Tumor Microdialysis Study. Examination of the TNP-470:TMZ combination by the microdialysis technique addressed the same primary questions presented for the above tumor homogenate study: essentially, does TNP-470 therapy reduce TMZ tumor concentrations and to the same extent in V− and V+ glioma models? As mentioned above, the microdialysis method provides a measure of interstitial fluid:unbound drug concentrations and, thus, may be considered more indicative of tumor drug concentrations than drug measurements obtained from tumor homogenates that reflect the combination of vascular, interstitial fluid, and intracellular compartments. Analysis of dialysis fluid by HPLC does not require an extraction procedure as do tumor homogenates, permitting greater drug recovery and detection of lower drug concentrations. In fact, in our tumor homogenate study, the targeted steady-state TMZ plasma concentration was 40 μg/ml, as opposed to 20 μg/ml in the microdialysis investigations, to avoid potential problems measuring both TMZ and MTIC. Thus, the two technical approaches used in the s.c. models complimented one another to provide a comprehensive assessment of the tumor distribution of TMZ and MTIC.

TNP-470 decreased the rate of tumor growth over the five-dose course of treatment compared with vehicle control in both the V− and V+ treatment groups. Tumor size decreased by a mean of 10% in the V− TNP-470 group compared with an increase of 44% in the V− control group. Tumor size increased by 1.9% in the V+ TNP-470 group compared with an increase of 85% in the V+ control group. Body weight increased in both the V− and V+ control groups by 4.2% and 11%, respectively. TNP-470 treatment had a minimal effect on body weight, resulting in a reduction of 1.2% in the V− group and an increase of 1.6% in the V+ group. Collectively these data indicated TNP-470 was effective in retarding tumor growth, and that V+ tumors grew more rapidly, consistent with the role of VEGF on angiogenesis.

Table 3 provides physiological and pharmacokinetic data for the microdialysis study in s.c. tumors. Steady-state TMZ plasma concentrations ranged from a mean of 22.5 ± 1.5 μg/ml to 27.7 ± 7.2 μg/ml in all treatment groups and were not statistically different from one another. In animals bearing V− tumors, tumor interstitial fluid TMZ concentrations were a mean of ~12 μg/ml in both control- and TNP-470-treated groups ($P > 0.05$). There was a definite reduction ($P = 0.076$) in TMZ tumor interstitial fluid concentrations in TNP-470-treated (*i.e.*, mean, 12.0 μg/ml) compared with control (mean, 16.6 μg/ml) in V+ tumors. This decrease was reflected in a significant reduction ($P = 0.028$) of ~29% in the mean steady-state tumor:plasma TMZ concentration ratio in the V+ TNP-470 treatment group compared with the V+ vehicle control group. The analogous V− groups had equivalent mean steady-state tumor:plasma TMZ concentration ratios of 0.49, and were nearly equivalent to the mean value of 0.52 in the V+ TNP-470 group. Comparison of the results from this microdialysis study to the above tumor homogenate study reveals an analogous effect of TNP-470 on TMZ tumor concentrations and the associated tumor:plasma concentration ratios. In the V+ model, the 30 mg/kg TNP-470 treatment regimen decreased TMZ tumor concentrations and tumor:plasma concentration ratios by ~30% compared with vehicle control. The microdialysis method measures unbound interstitial fluid TMZ concentrations and represents a fraction of the total tumor concentrations that are dependent on the extent of protein binding. We determined by an *in vitro* ultrafiltration method that TMZ was 30% plasma protein bound over a broad concentration range, and thus, the interstitial fluid unbound TMZ:total plasma concentration ratios should be on the order of 0.7, which is comparable with the range of 0.49–0.73 observed for all V− and V+ treatment groups. Similar to the tumor homogenate study, unbound interstitial fluid TMZ concentrations were not altered by TNP-470 in the V− model, consistent with low VEGF levels and limited angiogenesis.

Endothelial cell markers and identification of suitable surrogates for determination of angiogenesis inhibitor activity is an evolving area (19). The vast majority of *in vivo* trials have used MVD, although both the antibody and technique of quantitation have differed. Our goal in using MVD as a pharmacodynamic end point of angiogenesis inhibition was to provide an independent measure of their biological activity unrelated to measurement of TMZ concentrations. MVD could serve as a common reference to assess dose-dependent antiangiogenic activity of different agents in future drug combination studies. MVD was quantitated by image analysis in two peripheral regions of the s.c. tumor, and in the stroma within 100 μm of the leading edge of the tumor. Fig. 3 illustrates the mean MVD measurements for each vascular region and treatment group. When averaged over all three vascular regions, the MVD was 6.4 ± 4.8% in the V− control group compared with 7.3 ± 2.7% in the V− TNP-470 treatment group. When averaged over all vascular regions, the V+ TNP-470 treatment group had a reduced MVD of 11.8 ± 5.5% compared with a mean value of 16.9 ± 12.2% in the vehicle treatment group ($P = 0.16$). Analysis of each vascular region indicated there was a significant reduction in MVD in the stromal region of the V+ TNP-470 group compared with the stromal region of the control group (see Fig. 3). In

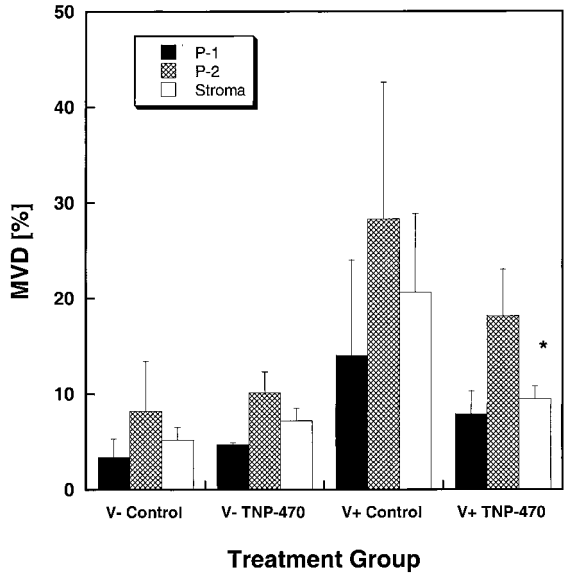

Fig. 3. MVD (*MVD [%]*) in s.c. gliomas. MVD measurements were obtained from three tumor vascular regions: peripheral region 1 (*P-1*), peripheral region 2 (*P-2*), and in stroma in vehicle control and TNP-470 30 mg/kg-treated nude rats. Tumors derived from either parental SF188 glioma cells (*V*−) or overexpressing VEGF SF188 glioma cells (*V*+). Tumors were collected 1 day after the last dose of a five-dose cycle of either TNP-470 or vehicle after administration of a steady-state infusion regimen of TMZ. ∗, significantly different from control at the $P < 0.05$ level; $n = 4$ or 5 animals/group.

all cases, MVD was greater in V+ tumors compared with the analogous regions in the V− tumors, consistent with the phenotypic alterations in the V− and V+ models (12). Collectively, these data indicate MVD was decreased at the tumor:stromal interface in V+ tumors, because of TNP-470 therapy, and support the use of MVD as a pharmacodynamic end point.

TMZ Interstitial Fluid Concentrations from Intracerebral Tumor Microdialysis Study. A basis to use TMZ as the anticancer drug in this series of studies was its favorable clinical activity in malignant glioma patients (11). It is known that drug uptake into brain tumors can be compromised by an intact BBB that may have a heterogeneous distribution throughout the tumor and coexist with tumor vasculature of high membrane permeability attributable to the breakdown of the BBB (20). For these reasons, examination of the TNP-470:TMZ combination in an intracerebral model should provide a more relevant assessment of the tumor disposition of TMZ in brain-tumor patients than the s.c. model. It was also decided to use only the V+ model in the intracerebral site, because there was not a TNP-470:TMZ interaction in the previous studies using V− s.c. tumors.

The study of drug distribution in brain-tumor bearing animals can make use of whole-tumor and microdialysis sampling. Whole-tumor extraction from brain can be problematic if tumor growth is highly infiltrative, making it difficult to demarcate normal brain and brain-tumor tissue. Microdialysis presents a similar type of sample integrity problem, in that the placement of the microdialysis probe is blinded, and its actual location in relation to the tumor can only be confirmed postmortem. Brain microdialysis has been criticized for the acute damage the probe may cause to the normal BBB (21), thereby compromising drug transport investigations. We have addressed these issues by our study design. The guide cannulas are inserted into brain approximately 10 days before the TMZ pharmacokinetic studies, allowing the initial damage to repair (21). Of course, any residual damage should be analogous in both control and TNP-470 groups, thus, permitting valid relative comparisons between treatment groups. The sampling issue is also partially accounted for by our study design by stereotaxic injection of the glioma cells in a geometrically defined region at the time the guide cannula is inserted. In our previous study (22), postmortem examination confirmed that this technique resulted in nearly a 100% success rate in microdialysis sampling from the tumor. We also confirmed the probe location by postmortem examination in the current study. Thus, each brain-tumor sampling methodology presents unique problems; however, based on our prior experience with brain-tumor microdialysis (22), it was chosen in this investigation.

Animal body weight at the time of the TMZ studies were 229.4 ± 29.4 g and 239.2 ± 32.9 g in the control and TNP-470 treatment groups, respectively. Brain-tumor MVD was significantly reduced in the TNP-470 group compared with control, being 8.4 ± 3.7% and 22.6 ± 13.0, respectively. These values are significantly different ($P = 0.019$), and support the use of MVD as a pharmacodynamic parameter for antiangiogenic therapy. The control- and TNP-470-treated intracerebral MVD values are within the range of values obtained for the different regions in the s.c. V+ tumors that were analogously treated (see Fig. 3).

Fig. 4 shows the individual mean steady-state TMZ plasma concentrations, the individual tumor interstitial fluid TMZ concentrations, and the tumor:plasma TMZ concentration ratios in the control and TNP-470 treatment groups. TMZ plasma concentrations ranged from 16.6 μg/ml to 28 μg/ml over both treatment groups, with means of 22.2 ± 4.5 μg/ml and 21.5 ± 3.1 μg/ml ($P > 0.05$) in control and TNP-470 groups, respectively. These values were very close to the targeted steady-state TMZ plasma concentration of 20 μg/ml. Mean tumor interstitial fluid TMZ concentrations obtained by the zero-flow microdialysis method were 8.6 ± 2.3 μg/ml and 4.2 ± 0.5 μg/ml in the control and TNP-470 groups, respectively. These TMZ tumor concentrations were significantly different ($P = 0.004$) between control and TNP-470 groups and of low variability, particularly in the TNP-470 group. The V+ s.c. (Table 3) tumor interstitial fluid TMZ concentrations were appreciably greater than the analogous intracerebral values, being about 16 μg/ml and 12 μg/ml in control and TNP-470 groups, respectively, and support an important role for the BBB in determining TMZ uptake in brain-tumors and provide additional confirmation of the importance of using the intracerebral model. Consistent with both the plasma and tumor TMZ concentrations in the

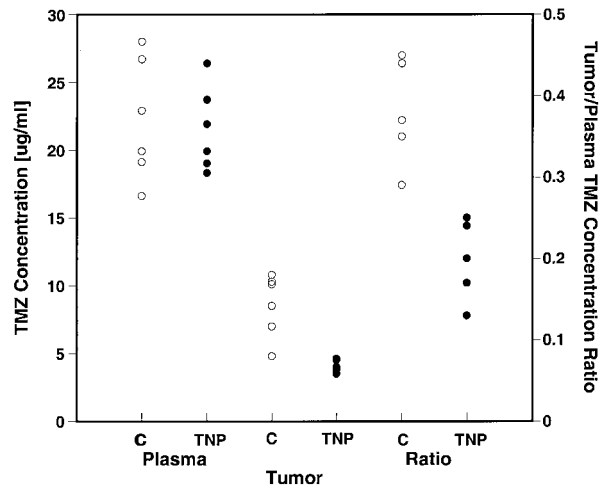

Fig. 4. Steady-state TMZ plasma and tumor interstitial fluid concentrations (*left, Y axis*) and the tumor:plasma TMZ concentration ratios (*right, Y axis*) in individual animals bearing intracerebral overexpressing VEGF (*V+*) tumors treated with TNP-470 (*TNP*; ●) or vehicle control (*C*; ○). Tumors were collected 1 day after the last dose of a four- to six-dose cycle of either 30 mg/kg of TNP-470 or vehicle after administration of a 7-h steady-state infusion regimen of TMZ. There were six animals/group; some symbols overlap. Tumor interstitial fluid TMZ concentrations and the tumor:plasma TMZ concentration ratios were significantly reduced in the TNP-470 group compared with control ($P < 0.05$).

intracerebral model, there was a significant reduction ($P = 0.004$) in the tumor:plasma TMZ concentration ratio in the TNP-470 (mean, 0.20 ± 0.04) group compared with the control group (mean, 0.38 ± 0.06). Therefore, TNP-470 treatment resulted in a highly significant 50% reduction in TMZ uptake into V+ intracerebral tumors.

DISCUSSION

A fundamental premise of cancer chemotherapy is to use drugs that have different mechanisms of action or targets and, preferably, nonoverlapping toxicities. This is commonly seen in the numerous combination regimens of cytotoxic drugs that include DNA-alkylating agents, microtubule inhibitors, and topoisomerase inhibitors. An extension of this principle applies to drugs that do not target tumor cells, but rather the tumor vasculature, such as angiogenesis inhibitors. Thus, a recent and logical chemotherapeutic strategy is the combined use of angiogenesis inhibitors and cytotoxic agents, because they possess different cellular targets and nonoverlapping toxicities (2–4, 23).

A small number of combination studies of angiogenesis inhibitors and cytotoxic drugs have been completed in animal models (2–4). On the basis of end points of efficacy, such as delay in tumor growth, combinations of cytotoxic drugs and angiogenesis inhibitors have, in general, shown additive activity in the preclinical models. Clinical trials of angiogenesis inhibitors as single agents and in combination with cytotoxic drugs are beginning to emerge (24).[4] In all of the reported combination trials, there has been no systematic effort to examine how angiogenesis inhibitors and cytotoxic agents might interact pharmacokinetically or pharmacodynamically and whether tumor drug concentrations might be adversely affected. The current investigation has begun to address these deficiencies by characterizing the disposition of TMZ in tumors in the absence and presence of an angiogenesis inhibitor.

The pharmacokinetic approach used throughout this investigation was to achieve a steady-state condition or constant TMZ plasma and tissue concentrations. As compared with a single-dose TMZ study design in which concentrations would be time-dependent, the steady-state design had two distinct advantages. One, the steady-state design minimized the number of animals required to characterize the tumor disposition of TMZ by calculation of steady-state tumor:plasma drug concentration ratios. A single-dose design would require multiple timed samples to permit estimation of the tumor distribution of TMZ by the ratio of the tumor:plasma areas under the TMZ concentration-time curve. The single design would require serial sacrifices to measure both TMZ and MTIC areas under the concentration-time curves in tumor homogenates. The second benefit of the steady-state design was that it enabled the zero-flow microdialysis calibration method to be used, which eliminated the need of a retrodialysis calibrator commonly used in single-dose designs, eliminating inaccuracies due to selection of a calibrator possessing different membrane permeability characteristics than TMZ.

On the basis of the pivotal role of VEGF to the grade and progression of tumors (19, 25–30), we evaluated the TNP-470:TMZ combination in our previously developed xenograft models that used isogenetic glioma cells differing in VEGF expression (12). It was clear that, in V− tumors, there were no alterations in the tumor disposition of TMZ consistent with the limited neovascularization, the lower MVD, and the minimal effect TNP-470 would have on endothelial cell permeability. On the contrary, TNP-470 caused appreciable reductions in tumor concentrations of TMZ in both s.c. and intracerebral V+ tumors. The active metabolite of TMZ, MTIC, also suffered large reductions in its tumor concentrations in the presence of TNP-470.

The mechanisms that underlie the changes in TMZ tumor concentrations in the presence of TNP-470 therapy are unknown. It is possible that changes in MVD, membrane permeability, and even tumor blood flow could play a role. Membrane transport phenomenon would seem to indicate that TMZ could traverse the hyperpermeable capillary (*i.e.*, V+ model) by both passing through adjacent endothelial cell gaps (*i.e.*, a paracellular route) and through the lipoidal cell membrane (*i.e.*, a transcellular route). Because TMZ is lipophilic and not known to undergo any specialized membrane transport processes, the transcellular route is simply the normal diffusion pathway, unlike the various endocytic pathways used by macromolecules. Paracellular transport of TMZ through hyperpermeable capillaries could represent an additional means of tumor uptake that is not available in normal tissues, such as in brain with an intact BBB. This pathway may be eliminated in hyperpermeable tumors after successful antiangiogenic therapy. An additional explanation for reduced TMZ tumor concentrations in the presence of TNP-470 could be a reduction in MVD and the associated decrease in the capillary surface area available to TMZ. This mechanism would seem more critical for drugs that undergo specialized and saturable transport processes. Finally, another potential contributory factor to the action of TNP-470 on TMZ tumor concentrations is based on recent findings that angiogenesis inhibitors can alter tumor blood flow with both increases and decreases observed (31, 32). Because lipophilic drugs such as TMZ may be subject to blood flow-limited tissue uptake, it is possible that the reduced tumor concentrations of TMZ in the presence of TNP-470 in the V+ model reflect a reduction in tumor blood flow. The likelihood of this mechanism will require an independent measure of tumor blood flow. Regardless of the mechanisms responsible for reduced tumor concentrations of TMZ in the presence of TNP-470, it was shown by two different methodologies that these changes can be significant. On the basis of the study design of delaying TMZ administration 1 day after the final dose of TNP-470, the effects of TNP-470 on tumor concentrations of TMZ are pharmacodynamically mediated rather than attributable to a direct pharmacokinetic interaction, because TNP-470 and metabolites would be eliminated before TMZ administration. It will be important to consider pharmacokinetic interactions, such as those recently shown *in vitro* concerning metabolic interactions (33) between angiogenesis inhibitors and cytotoxic drugs, so that a comprehensive assessment of potential drug interactions can be made.

Given the strong rational and favorable preclinical results for combination cytotoxic drug and antiangiogenic therapy, the drug interaction observed here suggests that treatment regimens may not be optimal. A simple solution of increasing the cytotoxic drug dose proportional to the reduction in tumor concentrations (~50% for TMZ in brain-tumors) is undesirable because of the likely increase in dose-dependent toxicity. Much more pharmacokinetic and pharmacodynamic information will be needed for both classes of agents to fully explore how combination drug dosing regimens can be designed to minimize any deleterious effect attributable to the angiogenesis inhibitor. Factors to be considered will include the time-dependent changes in the angiogenesis inhibitors pharmacodynamic effects. In the current investigation, the pharmacodynamic action of TNP-470 on capillaries and the associated reductions in TMZ tumor concentrations were clearly established after five doses. How rapidly this action might be reversed, permitting a therapeutic dose of a cytotoxic agent without appreciably influencing tumor growth, is unknown. Recent reports indicate that alternate dosing schedules, referred to as metronomic dosing, of cytotoxic agents allow them to function as angiogenesis inhibitors (34). Whether alternate dosing schedules of angiogenesis inhibitors can be designed that minimize potential reductions in cytotoxic drug uptake requires additional investigation. Identification of optimal combination dosing regimens will require a pharma-

cokinetic-pharmacodynamic framework. In conclusion, in a preclinical tumor model that overexpressed VEGF, it was found that TNP-470 reduced tumor concentrations of TMZ. Additional work is warranted to develop pharmacokinetic-pharmacodynamic strategies to identify optimal combination regimens of angiogenesis inhibitors and cytotoxic drugs.

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