Uptake of Temozolomide in a Rat Glioma Model in the Presence and Absence of the Angiogenesis Inhibitor TNP-470¹

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Abstract

The angiogenic phenotype is associated with hyperpermeable capillaries. Through treatment with angiogenesis inhibitors capillary permeability may be reduced, and it can be anticipated that cytotoxic agents coadministered may be adversely affected. The current investigation examined this possibility for the combination of TNP-470, an angiogenesis inhibitor, and temozolomide (TMZ), a DNAalkylating agent with demonstrated activity in brain tumors. TNP-470 (30 mg/kg) was given s.c. on days 6, 8, 10, 12, and 14 following s.c. implantation of rat C6 glioma cells in Sprague-Dawley rats. On the 15th day following tumor implantation, control (no TNP-470) and treated rats received 40 mg/kg of TMZ intrasentially. Prior to dosing, a linear microdialysis probe was placed in the tumor to collect interstitial fluid. Plasma and interstitial fluid samples were collected for 8 h and measured for TMZ by a high-performance liquid chromatography assay. Pharmacokinetic parameters for TMZ were calculated by noncompartmental methods. Total systemic clearance (39.8 ± 7 versus 44.2 ± 14 ml min⁻¹ kg⁻¹) and volume of distribution (5.4 ± 2 versus 5.2 ± 0.8 L kg⁻¹) were not significantly different in control and TNP-470-treated animals. However, the mean TMZ area under the interstitial fluid concentration-time curve was reduced by 25% in the TNP-470-treated group compared to the control (5450 ± 1892 versus 4120 ± 1790 µg min ml⁻¹; P < 0.05). It appears that TNP-470 caused this reduction in the tumor uptake of TMZ by its pharmacodynamic action on the tumor vasculature. Since combination regimens using angiogenesis inhibitors and cytotoxic drugs will be increasingly used, pharmacokinetic and pharmacodynamic investigations will be needed to determine how such combinations can be used effectively. The current animal model, which utilized tumor microdialysis, can serve as a model to further analyze combination chemotherapy.

Introduction

The angiogenic phenotype is composed of a cascade of biochemical and cellular events that cause tumors to be invasive and malignant (1–5). Within this cascade, a variety of growth factors are secreted that result in endothelial cell proliferation and neovascularization. The dynamic neovascularization process is associated with a hyperpermeable state, as demonstrated with colloids and macromolecules (6–8). Since transvascular exchange is comprised of both convective and diffusion mechanisms (9), the possibility exists of angiogenesis also influencing the uptake of small molecular weight cytotoxic agents. Treatment of solid tumors with angiogenesis inhibitors, by inhibition of neovascularization, may cause a reversion from a hyperpermeable state to a normal vascular permeability state. In this case, cytotoxic drugs coadministered with angiogenesis inhibitors may exhibit a reduction in their tumor uptake due to a reduction in capillary permeability. Tumor capillary permeability is dependent on both capillary structure and action of cytokines (2, 6, 7). One such cytokine, VEGF³ has been implicated in malignant gliomas and has been correlated to the tumor grade (4, 10–13). A hypothesis can be forged that alterations in cytotoxic drug uptake into tumors due to angiogenesis inhibitors may be related to the expression of VEGF and its tyrosine kinase receptors, flk-1 and flt-1 (14–16).

The current study examined the disposition of temozolomide, a drug highly active in brain tumors (17, 18), in the absence and presence of TNP-470, a fumagillin analogue, that inhibits endothelial cell proliferation (19–21). To facilitate the evaluation of this proposed drug interaction, a s.c. rat C6 glioma animal model that used tumor microdialysis to provide interstitial fluid TMZ concentrations was used. The C6 glioma model is considered an appropriate model for angiogenesis due to up-regulation of VEGF and its cognate receptors, flt-1 and flk-1 (22).

Materials and Methods

Materials. Rat C6 glioma cells were obtained from American Type Culture Collection (CCL107; Rockville, MD). Temozolomide was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). TNP-470 was generously supplied by TAP Pharmaceuticals (Deerfield, IL). Metronidazole was obtained from Aldrich (Milwaukee, WI). FITC-dextran (M, 70,000) was obtained from Molecular Probes, Inc. (Eugene, OR). Linear microdialysis probes (MD-2000) were purchased from Bioanalytical Systems (West Lafayette, IN). Male Sprague-Dawley rats were purchased from Taconic Farms, Inc. (Germantown, NY). HPLC analyses of samples were performed with a Hewlett Packard Model 1050 liquid chromatographic system (Sunnyvale, CA). All other chemicals and supplies were obtained from commercial sources.

Implantation of C6 Glioma Cells. Rat C6 glioma cells were grown in Ham’s FIO medium with 15% horse serum and 2.5% fetal bovine serum as a monolayer and were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The glioma cells were trypsinized (0.04% trypsin), centrifuged, and resuspended at a concentration of 3 × 10⁶ cells/0.5 ml in Ham’s FIO medium. For s.c. transplantation, animals were briefly anesthetized with ether. Glioma cells (3 × 10⁶) suspended in 0.5 ml of culture medium were slowly injected s.c. in the dorsal neck region. After cell implantation, animals were returned to their cages and received standard rat diet and water ad libitum.

Confocal Microscopy Study. Vascular permeability in the tumor was assessed in control and TNP-470-treated rats with dextran (M, 70,000) labeled with FITC (FITC-dextran). Animals briefly anesthetized with ether were administered 30 mg/kg of TNP-470 s.c. every other day (days 6, 8, 10, 12, and 14), starting from 6 days after the tumor implantation. Animals treated as control were administered vehicle alone that consisted of 1% ethanol and 5% gum arabic in normal saline. Twenty-four and 72 h after the last treatment, control and TNP-470-treated animals were anesthetized and the FITC-dextran injected i.v. through a jugular vein cannula. Ten min after injecting the dextran, the s.c. tumor was quickly removed and immediately frozen in liquid nitrogen. The frozen samples were kept at −70°C. The tumor was sectioned (10-µm sections) on a cryostat and examined with a confocal laser scanning fluorescent microscope.

Microdialysis Study. Animals briefly anesthetized with ether were administered 30 mg/kg of TNP-470 s.c. on days 6, 8, 10, 12, and 14 following tumor

1The abbreviations used are: VEGF, vascular endothelial growth factor; HPLC, high-performance liquid chromatography.

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implantation. Control animals were treated as the TNP-470 group, only they received vehicle alone. On the day following the last treatment (15th day), all animals 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); then a a right common carotid artery cannula was implanted. Next, a linear microdialysis probe was placed into the s.c. tumor as follows. A fiber attached to the probe was threaded through the eye of a small needle that was passed through the tumor. The probe was positioned so that the center of the membrane (10 mm) portion corresponded to the center of the tumor. The probe was anchored in place using glue on either side of the tubing where it exited the skin. The probe was connected to a syringe pump and perfused with a solution of metronidazole (10 µg/ml in Ringer’s solution) at a flow rate of 2 µl/min. TMZ (40 mg/kg) was dissolved in 15% DMSO just prior to administration intraarterially over a period of 3 min. All surgeries were performed under aseptic conditions.

Sample Collection. After TMZ administration, the dialysate from the microdialysis probe was collected every 20 min for the first 5 h and then every 30 min for the next 3–4 h in a vial containing 1 ml HCl (5 µl–7.5 µl). Blood samples (150 µl) were collected from the arterial cannula into heparinized tubes at 10, 20, 30, 45, 60, 120, 180, 240, 360, and 480 min after TMZ administration. The heparinized blood was centrifuged at 4°C to obtain plasma and immediately transferred to a vial containing 20 µl of 1 N HCl. The acidified plasma and dialysate samples were then stored at −80°C until further analysis by HPLC. After the last blood sample was collected, animals were euthanized, and tumors were removed and fixed in 10% formalin.

Sample Preparation and Analysis. Plasma (100 µl) samples containing 20 µl of 1 N HCl were transferred to a microcentrifuge tube containing one volume (120 µl) of chilled acetonitrile. The contents of the tube were vortexed, and the proteins precipitated by centrifugation at 10,000 rpm for 2 min. The resultant 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 were injected onto the HPLC. Dialysate samples (10 µl) were injected directly onto the HPLC.

Chromatographic separation was achieved using a mobile phase flow rate of 0.7 ml/min on an Alltech Spherisorb CN (150 mm × 4.6 mm, 5 µm). TMZ was detected at 323 nm 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 in vivo recovery method using metronidazole, a retrodialysis marker (23).

Noncompartmental analysis was used to calculate pharmacokinetic parameters of TMZ. For each animal administered TMZ, the area under the plasma, interstitial fluid concentration-time curve (AUCp, or AUCp), and the first nonnormalized moment (AUMCp) in plasma were determined by NCOMP (24). The following pharmacokinetic parameters were estimated for TMZ in the presence and absence of TNP-470:

\[
CL = \frac{Dose}{AUC_{p}}
\]

\[
MRT = \frac{AUMC_{p}}{AUC_{p}}
\]

\[
V_{ss} = (CL)(MRT)
\]

\[
t_{1/2} = \frac{0.693}{K}
\]

where \(CL\) is the total systemic clearance and \(V_{ss}\) is the volume of distribution at steady state, \(t_{1/2}\) is the elimination half-life, and \(K\) is the terminal disposition rate constant.

A Mann-Whitney test was used to determine significant differences in the measured parameter in the absence and presence of TNP-470. A value of \(P < 0.05\) was considered statistically significant.

Pharmacodynamic Studies. Animal body weight was measured for each treatment. The final tumor size was calculated as:

\[
\text{Tumor volume} (\text{cm}^3) = 0.5ab^2
\]

where \(a\) is the longest diameter and \(b\) is the shortest diameter. The effect of each treatment on tumor volume was represented as:

\[
\%T/C = \frac{\text{Mean tumor volume of TNP-470 treated group}}{\text{Mean tumor volume of control group}} \times 100
\]

The capillary density was evaluated by an immunohistochemical method for measurement of factor VIII. Tissue sections from paraffin-embedded blocks were biotin complex method (25). Central and peripheral blood vessels in a vascularized region of the tumor section were counted in five ×200 fields.

Results

A quantitative analysis of tumor vasculature demonstrated that the mean number of tumor vessels, in the control and TNP-470-treated groups, in the periphery of the tumor section was \(9 \pm 1 (n = 5)\) and \(4 \pm 3 (n = 5)\) vessels, respectively (Fig. 1). However, the mean number of vessels in the center of same tumor section in both the groups was not significantly different. The mean tumor volume was 2490 ± 1672 mm\(^3\) (\(n = 6\)) and 1013 ± 275 mm\(^3\) (\(n = 5\)) for control and treated groups, respectively. Treatment with TNP-470 resulted in a T/C of 41%.

Confocal micrographs show intense and diffuse staining due to FITC-dextran in tumor tissue in the control versus TNP-470-treated group, indicating that capillary permeability was altered. Quantitation of light intensity was slightly greater in the middle versus periphery of the tumor sections in both groups (control versus TNP-470) 24 h after the last treatment. Also, the light intensity averaged over the whole tumor was decreased in TNP-470-treated animals. The pattern of accumulation of FITC-dextran in tumor tissue varied depending on the

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Fig. 1. Immunohistochemical detection of Factor VIII in control (A) and TNP-470-treated (B) tumors. A, a large number of positively stained small capillary blood vessels. B, the relative decrease of the factor VIII-positive capillary contours after treatment with the angiogenesis inhibitor drug TNP-470. Both micrographs were taken in the periphery of the tumors. Immunohistochemical stain using diaminobenzidine and light counterstain with hematoxylin (×100).
number of days after inoculation of tumor cells for TNP-470 (data not shown).

Fig. 2 shows mean TMZ plasma concentration-time profiles in the absence and presence of TNP-470. Pharmacokinetic parameters for TMZ for control and TNP-treated groups are presented in Table 1. Total systemic clearance (39.8 ± 7 versus 44.2 ± 14 ml min⁻¹ kg⁻¹) and volume of distribution at steady-state (5.4 ± 2 versus 5.2 ± 0.8 L kg⁻¹) were not significantly different in control and TNP-470-treated animals.

The mean concentrations of TMZ in tumor interstitial fluid versus time in the presence and absence of TNP-470 are shown in Fig. 3. The mean TMZ area under the interstitial fluid concentration-time curve (AUC₁ᵢ) was 5450 ± 1892 μg min ml⁻¹ (control) and 4120 ± 1790 μg min ml⁻¹ (TNP-470) and was significantly different (P < 0.05, Mann-Whitney test). The AUCᵢ/CLUD ratio was found to be approximately 5.6 ± 2.7 and 4.1 ± 0.5 for control and TNP-treated rats and was not significantly different. Although not statistically significant, the effect of TNP-470 on the AUCᵢ/CLUD ratio is analogous to that on AUCᵢ values. This ratio also indicates that TMZ extensively distributed into tumor tissue, consistent with the results obtained by Tsang et al. (26). MTIC was not detected in the rat plasma and in tumor tissue of rats that had received TMZ due to sample collection conditions.

TNP-470 treatment resulted in a significant weight loss (229 ± 37 g versus 314 ± 27 g; n = 6), a toxicity described previously (20).

**Discussion**

Combination chemotherapy of angiogenesis inhibitors and cytotoxic agents is a new therapeutic strategy that is directed at two fundamentally different targets: (a) the tumor cell via cytotoxic agents; and (b) the process of angiogenesis that offers numerous points of inhibition. The combination of TMZ and TNP-470 are representative of a likely therapeutic combination, particularly for brain tumors, based on their demonstrated clinical activity to date (17, 18, 27). TMZ is a produg that is initially converted to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, which undergoes further molecular conversions to form DNA-alkylating species. It is of low molecular weight (i.e., Mᵣ 194,000) and is presumed to cross biological membranes by passive diffusion. Thus, in terms of its physicochemical properties and biological actions, it serves as a model agent for other small cytotoxic molecules.

TNP-470, although not devoid of cytotoxicity to tumor cells, its development as an angiogenesis inhibitor is based on its potent ability to selectively inhibit endothelial cell proliferation (19). In this study, TNP-470 caused significant changes in tumor microvasculature in C6 glioma-bearing rats. The number of the vessels in the periphery of the tumor in TNP-470-treated rats were markedly decreased compared to the untreated rats. In summary, the combination of TMZ and TNP-470 is a model system to assess potential interactions between angiogenesis inhibitors and cytotoxic agents.

A number of preliminary studies were conducted in the current investigation to determine the schedule of drug treatments in relation to the time of tumor implantation. Although some preclinical models initiate antiangiogenic therapy at the time of tumor implantation to maximize tumor growth retardation, this is neither clinically relevant nor practical in terms of placement of the microdialysis probe. The 5-day delay in initiation of TNP-470 treatment enabled the tumor to grow to a size that facilitated placement of the microdialysis probe. Under the current multiple-dose TNP-470 regimen (30 mg/kg every other day for five doses), capillary permeability to FITC-dextran was reduced in the periphery regions of the C6 tumor compared to untreated control. Permeability to FITC-dextran was slightly increased in the middle of the tumor compared to control, yet averaged over all tumor sections, permeability was decreased in TNP-470-treated animals. Kato et al. (20) had shown that the early initiation of a prolonged fractionated dosing schedule, analogous to ours, of TNP-470 was more effective than a short-term high-dose treatment or a delayed treatment schedules. The TMZ dose (40 mg/kg) used in this study was reported not to cause toxicity in mice (26) and was optimally effective against the TLX5 lymphoma when given on 5 consecutive days.

The current investigation was formulated on the hypothesis that cytotoxic drug tumor uptake will be reduced in the presence of angiogenesis inhibitors due to the latter’s effect on capillary permeability. Based on the drug treatment schedules and the lack of changes in macropharmacokinetic properties of TMZ, the observed inhibition of TMZ uptake due to TNP-470 is pharmacodynamically mediated. TMZ was administered 24 h after the last dose of TNP-470, a period of time that would allow TNP-470 and its active metabolites to be eliminated (28). Thus, the reduction in TMZ tumor uptake in the presence of TNP-470 therapy is most likely due to a pharmacodynamic effect of TNP-470 on capillary permeability. It is not known how these changes may occur; however, the likely mechanisms are related to structural changes (i.e., elimination of endothelial cell gaps, or altered membrane components) in the microvasculature and/or the inhibition of the VEGF pathway, with both processes possibly being interdependent. VEGF is presumed to enhance tumor capillary permeability by increasing the functional activity of vesicular-vacuolar

**Table 1** Pharmacokinetic parameters for temozolomide in C6 glioma-bearing rats following an intraarterial dose of 40 mg/kg in the presence and absence of an angiogenesis inhibitor, TNP-470

<table>
<thead>
<tr>
<th>Temozolomide pharmacokinetic parameters</th>
<th>Control (No TNP-470)</th>
<th>Treated (TNP-470)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCᵢ/CLUD µg ml⁻¹ min</td>
<td>5450 ± 1892</td>
<td>4120 ± 1790</td>
</tr>
<tr>
<td>AUCᵢ µg ml⁻¹ min</td>
<td>1040 ± 253</td>
<td>1000 ± 386</td>
</tr>
<tr>
<td>AUCᵢ/CLUD µg ml⁻¹</td>
<td>5.59 ± 2.7</td>
<td>4.05 ± 0.45</td>
</tr>
<tr>
<td>CL ml min⁻¹ kg⁻¹</td>
<td>39.8 ± 7</td>
<td>44.2 ± 14</td>
</tr>
<tr>
<td>Vᵢ/CLUD ml kg⁻¹</td>
<td>5340 ± 2058</td>
<td>5240 ± 778</td>
</tr>
<tr>
<td>tᵢ/CLUD minutes</td>
<td>86 ± 58</td>
<td>100 ± 47</td>
</tr>
</tbody>
</table>

* Significantly different in control and treated groups; P < 0.05.
organelles that may open fenestrae and enable extravasation of macromolecules (29, 30). Inhibition of endothelial cell proliferation by TNP-470 and a resultant reordered vasculature may reduce extravasation of epidermal growth factor and, accordingly, secretion of VEGF from tumor cells (12). These suggested interrelated actions may alter capillary permeability and, consequently, drug uptake.

Recently, Teicher et al. (31) has reported increased total tumor concentrations of [14C]cyclophosphamide and cis-diaminedichloroplatinum at 6 h in the presence of an antiangiogenic regimen of TNP-470 and monocycine in mice. The differences in study design that may be relevant were single 6-h measurements of total tumor drug concentrations, lack of pharmacokinetic data, and a different tumor model. In particular, measurement of total tissue drug concentrations not only masks concentration differences in the vascular and extravascular compartment but also potential regional differences in drug concentration at the center and periphery of the tumor. In fact, based on the more central microdialysis probe placement used in the current study, we may have underestimated reductions in cytotoxic drug uptake in the tumor periphery, the site of active neovascularization. In addition, without knowing possible alterations in the pharmacokinetics of the cytotoxic agent in the presence of antiangiogenic therapy, one cannot attribute elevations in tumor concentrations to tumor-specific events, rather these could result from elevated plasma concentrations. Clearly, the combined use of serial plasma and interstitial fluid sampling allows one to differentiate the “plasma” pharmacokinetics of TMZ from those in the tumor. In fact, the reduction in TMZ tumor interstitial fluid concentrations may have gone undetected without the use of microdialysis.

Evaluation of interactions between angiogenesis inhibitors and cytotoxic agents will require pharmacodynamic data to fully assess their importance. In this regard, various efficacy trials of combinations of angiogenesis inhibitors and cytotoxic drugs in preclinical models have been reported (20, 31, 32). Many of these show additive or synergistic effects on tumor size, tumor growth delay, and number of metastases. As specific biochemical measures of drug toxicity are attained and additional kinetic data are generated, these interesting findings can be understood. In our model, we would predict less DNA damage due to TMZ in the presence of TNP-470.

Possibly more than anything else, the current study demonstrates the need to further explore the pharmacokinetic and pharmacodynamic basis of combination chemotherapy. It is only through such explorations that the optimal use of such combinations will be realized.

Acknowledgments

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References


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