Antiangiogenic and Antitumor Effects of a Protein Kinase Cβ Inhibitor in Human T98G Glioblastoma Multiforme Xenografts

Beverly A. Teicher,1 Krishna Menon, Enrique Alvarez, Elizabeth Galbreath, Chuan Shih, and Margaret Faul
Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285

ABSTRACT

Although rare, the morbidity and mortality from brain tumors are significant. Chemotherapy has made only a small impact on these tumors. The human T98G glioblastoma multiforme cell line was used as a brain tumor model. The protein kinase Cβ inhibitor 317615·2HCl was not highly cytotoxic toward T98G cells in culture and was additive in cytotoxicity with carmustine (BCNU). When nude mice bearing s.c. T98G tumors were treated with 317615·2HCl p.o. twice daily on days 14–30 after tumor cell implantation, the number of intratumoral vessels stained by CD31 was decreased to 37% of control, and the number of intratumoral vessels stained by CD105 was decreased to 50% of control. The compound 317615·2HCl was an active antitumor agent against s.c. growing T98G xenografts. A treatment regimen administering 317615·2HCl before, during, and after BCNU was compared with a treatment regimen administering 317615·2HCl sequentially after BCNU. In the tumor growth delay determination of the s.c. tumor, the sequential treatment regimen was more effective than the simultaneous treatment regimen. However, when the same treatments were administered to animals bearing intracranial T98G tumors, the survival of animals receiving the simultaneous treatment regimen increased from 41 days for those treated with BCNU alone to 102 days for animals treated with the combination, whereas animals receiving the sequential treatment regimen survived 74 days. Treatment with the protein kinase Cβ inhibitor decreased T98G glioblastoma multiforme angiogenesis and improved treatment outcome with BCNU.

INTRODUCTION

There are at least 15,000 new cases of primary brain and CNS2 malignant neoplasms diagnosed in the United States per year. The most common malignant CNS tumor is glioblastoma multiforme. The mean age of patients with glioblastoma multiforme is 52 years. These tumors cause approximately 11,000 deaths per year. Despite the relatively small numbers of CNS tumors, the morbidity and mortality they cause are significant (1). Surgery remains the primary treatment for CNS tumors. After surgery, patients with glioblastoma multiforme or anaplastic astrocytoma are treated with radiation therapy. Chemotherapy is used adjuvantly with surgery and radiation therapy. The most commonly used chemotherapeutic agents are the nitrosoureas including BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and streptozotocin. The platinum complexes cisplatin and carboplatin are also used. Most recently, temozolomide has been approved in the United States for treatment of astrocytoma and is in clinical trial for other CNS tumor indications (2–4).

Most solid tumors increase in mass through the proliferation of malignant cells and stromal cells including endothelial cells, leading to formation of a tumor vasculature (5). Because active angiogenesis is a critical component of the mass expansion of most solid tumors, this process is a valid target for therapy (6). Angiogenesis (vasculature formation) during malignant growth is a complex process. Elucidation of the process has involved recognition of angiogenic stimuli such as hypoxia and nutrient deprivation; recognition of angiogenic factors produced by malignant cells, fibroblasts, and tumor-infiltrating leukocytes; and recognition that there may be a concomitant decrease in negative angiogenic regulators by the same three cell populations within the tumor for angiogenesis to occur (6–10). Preclinical and clinical studies have shown that malignant cells in culture and tumors in vivo can and most often do express an array of angiogenesis stimulators and negative regulators. Clearly, angiogenesis is a highly complex and closely regulated process, and it is not surprising that vasculature in malignant masses is often poorly formed, irregular, lacking complete structure, and inadequate to feed the tissue (11–13). The combination of certain antiangiogenic agents with standard therapies appears to be synergistic (14).

The most clear-cut, direct-acting, and most frequently found angiogenic factor in cancer patients is VEGF (5–8, 15, 16). VEGF expression has been associated with primary breast cancer, brain tumors, cervical neoplasias, lung cancer, stomach cancer, colon cancer, and other types of cancer. Furthermore, up-regulation of the VEGF receptors, Flt-1 and KDR, has been observed in tumor-associated endothelial cells in a variety of tumors including breast, brain, kidney, bladder, ovarian, and
colon tumors. The signal transduction pathways of the KDR/Flk-1 and Flt-1 receptors include tyrosine phosphorylation, activation of phospholipase C$_\gamma$, diacylglycerol generation, and phosphatidylinositol 3'-kinase with downstream activation of PKC, activation of the mitogen-activated protein kinase pathway (17–20), and possibly by translocation of PKC into the cell nucleus (21, 22).

PKC is a gene family consisting of at least 12 isoforms (23–25). Based on differing substrate specificity, activator requirements, and subcellular compartmentalization, it is hypothesized that activation of individual PKC isoforms preferentially elicits specific cellular responses (24, 26). To assess the contribution of PKC activation to VEGF signal transduction leading to neovascularization and enhanced vascular permeability, the effects of a PKCβ-selective inhibitor that disrupts the phosphotransferase activity of conventional and novel PKC isoforms via an interaction at the ATP-binding site was studied (26–30). The signal transduction from extracellular protein growth factors occurs by a variety of mechanisms that share many common features. Activation of specific receptor kinases does not activate unique intracellular kinases that then result in a linear signaling pathway; rather, multiple signaling cascades can be activated, producing combinatorial effects that allow more refined regulation of the biological outcome (31). The intracellular signal transduction pathways for VEGF and bFGF in endothelial cells have not been fully elucidated; however, it is likely that PKC is an important pathway component for both mitogens. Neangiogenesis in the eyes of rats bearing corneal micropocket implants of either VEGF or bFGF was inhibited by treatment of the animals with 317615 (30 mg/kg) p.o. twice per day (3). The human SW-2 small cell lung carcinoma growing in nude mice secretes VEGF and bFGF that are measurable in the plasma of the animals (317615:2HCl p.o. twice per day). The human SW-2 small cell lung carcinoma growing in nude mice secretes VEGF and bFGF that is measurable in the plasma of the animals. Treatment of SW-2-bearing mice with 317615:2HCl p.o. twice per day resulted in a countable decrease in intratumoral vessels and a corresponding slowing of tumor growth (3).

The current study was undertaken to examine the effect of the small molecule PKCβ inhibitor 317615:2HCl on intratumoral vessel development and the response of s.c. and intracranially implanted human T98G glioblastoma multiforme to BCNU.

**MATERIALS AND METHODS**

**Drugs.** BCNU was purchased from the Indiana University Clarion Health Medical Center pharmacy. The compound 317615:2HCl was prepared by Dr. B. A. Teicher, E. Alvarez, K. Menon, E. Considine, C. Shih, and M. Faul. Antiangiogenic effects of a protein kinase Cβ-selective small molecule, submitted for publication.

**Tumor Line.** The T98G cell line was purchased from American Type Culture Collection (Manassas, VA). The T98G glioblastoma multiforme line was derived from a 61-year-old male. The cells are hyperpentaploid with modal chromosome number of 128–132 (32).

**Cell Survival Analysis.** The T98G cells were grown in RPMI 1640 supplemented 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY). Cells grown in 25-cm$^2$ flasks to about 70% confluence were exposed to various concentrations of 317615:2HCl (0, 1, 5, 10, 50, 100, or 250 µM) for 5 h, exposed to various concentrations of BCNU (0, 1, 5, 10, 50, 100, or 250 µM) for 1 h, or exposed to 317615:2HCl (50 or 250 µM) for 5 h with various concentrations of BCNU during the third hour. After exposure to the agent or combination, the cells were washed with 0.9% PBS and suspended by exposure to 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at three or more dilutions for colony formation. After 7–10 days, the colonies were visualized by staining with crystal violet in methanol. Colonies of 50 cells or more were counted. The results are expressed as the surviving fraction of treated cells compared with control cultures.

**Intratumoral Vessel Counting.** Male 7–8-week-old nude mice (Charles River Laboratories, Wilmington, MA) were exposed to 4.5 Gy of total body radiation delivered using a GammaCell 40 irradiator (Nordion, Inc., Ottawa, Canada). Twenty-four h later, human T98G glioblastoma multiforme cells (5 × 10$^6$) prepared from a brei of several donor tumors were implanted s.c. in a 1:1 mixture of RPMI 1640 tissue culture media and Matrigel (Collaborative Biomedical Products, Inc., Bedford, MA) in a hind leg of the animals. The animals were treated with 317615:2HCl (30 mg/kg) p.o. by gavage twice per day on days 14–30 after tumor implantation. On day 35, the tumors (about 300 mm$^3$) were excised and submersed in Tissue-Tek (O.C.T. 4583 Compound; Sakura Finetek USA, Inc., Torrance, CA) on dry ice. The tissue blocks were stored at −80°C until processing. The tissues cut in 5-mm sections with a cryostat were fixed in cold acetone, dried, and stored at −80°C until stained. Sections were rinsed with 0.9% PBS three times and blocked with peroxidase blocking reagent and protein block (DAKO, Carpinteria, CA). Sections were then stained with 5 µg/ml anti-CD31 (PharMingen, San Diego, CA) or anti-CD105 (PharMingen) at room temperature for 30 min. Sections were rinsed and incubated with 2.5 µg/ml biotin-conjugated goat antirat antibody (PharMingen, San Diego, CA) for 10 min and then with peroxidase-conjugated streptavidin (DAKO) at 1:500 dilution for 10 min. Sections were developed with 3-amino-9-ethylcarbazole (AEC) substrate-chromogen system for 20 min at room temperature and counterstained with DAKO Mayer’s Hematoxylin (DAKO). The quantification of blood vessels was performed as described previously (33). The mean ± SE for 10 high-power fields were counted on 10 high-power fields (×200). The data are presented as the mean ± SE for 10 high-power fields.

**TGD Experiments.** Male nude mice were purchased from Charles River Laboratories at 5–6 weeks of age. When the animals were 7–8 weeks of age, they were exposed to 4.5 Gy of total body radiation delivered using a GammaCell 40 irradiator (Nordion, Inc., Torrance, CA). Twenty-four h later, human T98G glioblastoma multiforme cells (5 × 10$^6$) prepared from a brei of several donor tumors were implanted s.c. in a 1:1 mixture of RPMI 1640 tissue culture media and Matrigel (Collaborative Biomedical Products, Inc.) in a hind leg of the animals. In other mice, T98G cells (1 × 10$^3$) were implanted in 2 µl of media without serum intracranially. A midline scalp incision was made, and a hole was bored through the skull with a 27-gauge needle at a point 1 mm behind the right coronal suture and 1 mm lateral to the midline. The T98G cell suspension was injected into the right frontal lobe at
a depth of 1 mm from the dural surface. The needle was removed, and the hole was sealed with Nucast (Phamacal, Piscataway, NJ). The scalp was closed with a surgical clip (34).

Untreated s.c. T98G tumors grew to 500 mm³ in 31.4 ± 4.5 days. Animals were treated with 317615 2HCl (3, 10, or 30 mg/kg) p.o. by gavage twice per day on days 4–18 or on days 12–30 alone or with BCNU (15 mg/kg) by i.p. injection on days 7–11 after tumor cell implantation.

The progress of each s.c. tumor was measured twice per week until it reached a volume of 4000 mm³. As a general measure of toxicity, body weights were determined on the same schedule as tumor volume measurements. TGD was calculated as the time taken by each individual tumor to reach 1000 mm³ compared with the time taken in the untreated controls. Each treatment group included five animals. TGD times (measured in days) are the means ± SE for the treatment group compared with those for the control group (33, 35). Survival of animals was monitored daily. Animals that were moribund or unable to reach food or water were killed by carbon dioxide inhalation. Data for animals receiving intracranially implanted tumor cells are presented as mean survival (days) ± SE for the treatment group (34).

RESULTS

In cell culture, the compound 317615 2HCl was a more potent inhibitor of VEGF-stimulated human umbilical vascular endothelial cell proliferation (IC₅₀ = 150 nM, 72 h) than of human SW-2 small cell lung carcinoma cell proliferation (IC₅₀ = 3.5 μM, 72 h). The compound 317615 2HCl was not very cytotoxic toward human T98G glioblastoma multiforme cells in monolayer culture and had an IC₅₀ of ≥250 μM on 5 h of exposure (Fig. 1). Exposure of the T98G cells to BCNU for 1 h resulted in an IC₅₀ of about 250 μM. Exposure of the T98G cells to 317615 2HCl (50 μM) for 5 h with BCNU during the third hour resulted in an IC₅₀ for the combination of 2 μM, indicating primarily additivity of the agents.

Nude mice bearing human T98G glioblastoma multiforme growing as a s.c. xenograft on the thigh were treated with 317615 2HCl (30 mg/kg) p.o. twice daily on days 14–30 after tumor cell implantation. On day 31, tumors were collected and preserved in 10% phosphate-buffered formalin, and 5-mm-thick sections were immunohistochemically stained for expression of endothelial-specific markers, either CD31 or CD105. The number of intratumoral vessels in the samples was quantified by counting stained regions in 10 high-power microscope fields (×200). There was a 317615 2HCl-dependent decrease in the number of countable intratumoral vessels in the human T98G xenograft tumors. The number of intratumoral vessels stained by CD31 was decreased to 37% of the controls in animals treated with 317615 2HCl (30 mg/kg), and the number of vessels stained by CD31 was decreased to 50% of the controls in animals treated with 317615 2HCl (30 mg/kg; Fig. 2).

Simultaneous and sequential treatment regimens combining treatment with 317615 2HCl and the chemotherapeutic agent BCNU were studied in the human T98G glioblastoma multiforme using TGD as the end point when the tumor was implanted s.c. in the thigh and increase in life span as the end point when the tumor was implanted intracranially. For the simultaneous treatment regimen, 317615 2HCl was administered on days 4–18 after tumor implantation, and BCNU was administered on days 7–11 after tumor implantation. In this tumor model, treatment with 317615 2HCl on days 4–18 produced TGDs of 6.0 and 8.6 days at doses of 10 and 30 mg/kg, respectively (Fig. 3). Administration of the combination of 317615 2HCl (30 mg/kg) with BCNU resulted in a TGD of 15.2 days. There was no additional body weight loss in animals that received the 317615 2HCl with BCNU compared with animals receiving BCNU alone.

Nude mice that received an implant of T98G (10⁶ cells) intracranially had a mean survival time of 37 days. Administra-
tion of 317615-2HCl alone doubled the survival time of these animals, with 317615-2HCl (30 mg/kg) producing a mean survival time of 72 days (Fig. 4). Administration of BCNU to mice bearing the intracranial tumor implant resulted in a mean survival time of 41 days. The simultaneous combination of BCNU administration with 317615-2HCl produced mean survival times of 90 and 102 days at doses of 10 and 30 mg/kg, respectively.

The efficacy of administration of 317615-2HCl in the sequential treatment regimen after completion of BCNU therapy was also examined in s.c. and intracranially implanted human T98G glioblastoma multiforme. Over a dosage range from 3–30 mg/kg, treatment with 317615-2HCl alone on days 12–30 produced TGDs of 18.5–32.6 days, respectively, in the s.c. implanted tumor (Fig. 5). Sequential treatment with BCNU followed by 317615-2HCl (30 mg/kg) resulted in a TGD of 46.8 days. There was no additional body weight loss in animals that received BCNU followed by 317615-2HCl compared with animals that received BCNU only.

The effect of delaying treatment with 317615-2HCl from day 4 to day 12 after tumor implantation of intracranial human T98G glioblastoma multiforme was evident in the response of the intracranial tumor to therapy. Administration of 317615-2HCl over a dosage range from 3–30 mg/kg produced mean survival times in the animals from 57–72 days compared with 37 days for control animals (Fig. 6). Sequential treatment with BCNU followed by 317615-2HCl over the dosage range from 3–30 mg/kg resulted in mean survival times from 63–74 days compared with a mean survival time of 41 days after treatment with BCNU alone.

DISCUSSION

Marked neovascularization is a hallmark of many neoplasms in the CNS. Vascular pathology is a key feature glioblastoma multiforme characterized by hypervascularity, vascular permeability, and hypercoagulability. VEGF has been investigated as a potent mediator of brain tumor angiogenesis, vascular permeability, and glioma growth and is known to be up-regulated in most cases of glioblastoma multiforme (36–47). Microvessel density and VEGF levels have been shown to be independent prognostic markers of survival in fibrillary low-grade astrocytoma. Tumors with a larger number of microvessels also had a greater probability of undergoing malignant transformation (36). Another study examined the activated phosphorylated form of the KDR receptor in astrocytic neoplasms and found the phosphorylated form of KDR in fresh surgical specimens of glioblastomas (71%) and anaplastic gliomas (15%) but not in low-grade gliomas, indicating that the onset of angiogenesis is an important event during the disease progression of gliomas (42). Chan et al. (46) found the VEGF receptors, KDR and Flt-1, to be up-regulated in the tumor vasculature of glioblastoma multiforme, anaplastic oligodendroglia, and ependymomas with necrosis but not in grade II astrocytomas, anaplastic astrocytomas, or oligodendroglioma tumors. In meningiomas, VEGF was associated with both tumor vascularity and peritumoral edema (37). While looking for a correlation between angiographic neovascularization, peritumoral brain edema, and the expression of VEGF, Bitzer et al. (41) found that tumors with high VEGF staining had a significantly higher edema index and a higher edema incidence. In addition, all of the meningiomas with very high VEGF expression were associated with vascular tumor supply from cerebral arteries. Takano et al. (39) found that VEGF concentrations of glioblastoma cyst fluid were 200–300-fold higher than those of...
serum in the patients. VEGF concentration in the tumors was significantly correlated with the vascularity measured by counting vessels stained with von Willebrand factor antibody. VEGF is expressed in a wide spectrum of brain tumors and is associated with neovascularization. However, other angiogenic factors also appear to contribute to the vascularization of CNS neoplasms (38). The expression of angiopoietin-1 and angiopoietin-2 in human astrocytomas was investigated by in situ hybridization (45). Angiopoietin-1 mRNA was localized in tumor cells, and angiopoietin-2 mRNA was detected in endothelial cells. The results suggested that angiopoietins are involved in the early stage of vascular activation and in advanced angiogenesis and indicate that angiopoietin-2 may be an early marker of glioma-induced neovascularization. Takano et al. (48) investigated the expression of the angiogenic factor thymidine phosphorylase in human astrocytic tumors and found that thymidine phosphorylase was expressed in the tumor cells, macrophages, and endothelial cells. In another study, the mean concentrations of VEGF were found to be 11-fold higher in high-grade gliomas, and the mean concentrations of hepatocyte growth factor/scatter factor were found to be 7-fold higher in high-grade gliomas than in low-grade tumors (49). In addition, VEGF and hepatocyte growth factor/scatter factor appeared to be independent predictive parameters for glioma microvessel density. The findings of this study also suggested that bFGF is an essential cofactor for angiogenesis in gliomas.

The signal transduction from extracellular protein growth factors occurs by a variety of mechanisms that share many common features. Activation of specific receptor kinases does not activate unique intracellular kinases that then result in a linear signaling pathway; rather, multiple signaling cascades can be activated, producing combinatorial effects that allow more refined regulation of the biological outcome (31). The intracellular signal transduction pathways for VEGF and bFGF in endothelial cells have not been fully elucidated; however, it is likely that PKC is an important pathway component for both mitogens. Neoangiogenesis in the eyes of rats bearing corneal micropocket implants of either VEGF or bFGF was inhibited by treatment of the animals with 317615HCl p.o. twice per day resulted in a dose-dependent decrease in the number of countable intratumoral vessels in the tumors. The number of intratumoral vessels stained by factor VIII was decreased to one-half that of the controls in animals treated with 317615HCl (30 mg/kg), and the number of vessels stained by CD31 was decreased to one-quarter that of the controls in animals treated with 317615HCl (30 mg/kg). In the current study, a similar effect was observed in the human T98G glioblastoma multiforme grown as a s.c. xenograft tumor.

The potential of antiangiogenic agents to augment the antitumor activity of standard cytotoxic chemotherapeutic agents is becoming well established (14, 15). Among the antiangiogenic agents under investigation, TNP-470, an inhibitor of endothelial cell proliferation, has been shown to delay the growth of gliomas and other brain tumors in several studies (15, 50). The antiangiogenic combination of TNP-470 and minocycline increased the response of both intracranial and s.c. rat 9L gliosarcoma to BCNU or Adriamycin (34). Lund et al. (50) found that TNP-470 treatment increased the response of s.c. human U87 glioblastoma xenografts to radiation therapy but did not increase the response of intracranial U87 to radiation therapy. Angiostatin, an antiangiogenic internal fragment of plasminogen, has been shown to suppress the growth of rat C6 and rat 9L gliomas as well as human U87 glioma, regardless of whether tumor cells are implanted s.c. or intracranially (51). Angiostatin used in combination with fractionated radi-

![Fig. 4 Survival of animals bearing intracranial human T98G glioblastoma multiforme after treatment with 317615HCl (10 or 30 mg/kg) p.o. twice per day on days 4–18 alone or with BCNU (15 mg/kg, i.p.) on days 7–11. Data are the means of five animals. Bars, SE.](image1)

![Fig. 5 Growth delay of s.c. implanted human T98G glioblastoma multiforme after treatment with 317615HCl (3, 10, or 30 mg/kg) p.o. twice per day on days 12–30 alone or after administration of BCNU (15 mg/kg, i.p.) on days 7–11. Points are the means of five animals. Bars, SE.](image2)
eration therapy had a greater than additive effect on the growth of a human glioma in nude mice (52). The PKCβ inhibitor 317615-2HCl is a p.o. administered small molecule without toxicity in rodents at the antiangiogenic doses. The T98G glioblastoma multiforme after treatment with 317615-2HCl (3, 10, or 30 mg/kg) p.o. twice per day on days 12–30 alone or after administration of BCNU (15 mg/kg, i.p.) on days 7–11. Data are the means of five animals. Bars, SE.

**REFERENCES**


Antiangiogenic and Antitumor Effects of a Protein Kinase Cβ Inhibitor in Human T98G Glioblastoma Multiforme Xenografts


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/3/634

Cited articles
This article cites 51 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/3/634.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/7/3/634.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.