A multi-targeted tyrosine kinase inhibitor lenvatinib for the treatment of mice with advanced glioblastoma

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Abstract. Glioblastoma is the most aggressive primary brain tumor that originates from the glial cells in adults. Aberrant angiogenesis is essential for malignant glioblastoma tumorigenesis, development and metastasis. Lenvatinib is a multi-targeted anticancer agent that targets of receptor tyrosine kinases including vascular endothelial growth factor receptor 1 and 2, fibroblast growth factor receptor 1, platelet-derived growth factor receptor β and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog. In the present study, the therapeutic effects of lenvatinib as a treatment for glioblastoma were investigated in vivo and in vitro. The maximum dose toxicity (MDT) and treatment-associated adverse events of lenvatinib were identified by cytotoxicity assay in experimental mice. Increasing levels of the pro-apoptosis genes caspase-3, -8, -9 and -10 following lenvatinib treatment were determined by reverse transcription-quantitative polymerase chain reaction, and apoptosis of the malignant gliomas cells was analyzed by FACS. In vivo treatment with lenvatinib for BV-2 bearing male BALC/c nude mice was assessed via tumor growth suppression and long-term observation of survival. Subsequent cytotoxic T lymphocyte responses were further analyzed to determine the in vivo efficacy of lenvatinib treatment in mice with glioblastoma. The MDT of lenvatinib was identified as 0.24 mg, with relatively few side effects and improved efficacy in mice. Lenvatinib (0.24 mg) significantly increased apoptosis in BV-2, C6, BC3H1 and G422 glioma cell lines. Tumor growth was significantly inhibited and tumor-bearing mice demonstrated an improved survival rate following treatment with lenvatinib. In conclusion, lenvatinib provided an effective treatment outcome, and the results of the present study may help to achieve a comprehensive therapeutic schedule for clinical application.

Introduction

Glioblastoma is the most aggressive primary brain tumor that originates from the glial cells in adults or develops from existing malignant cells (1,2). Glioblastoma is characterized by the appearance of vascular proliferation, aggressive invasion and necrosis around human normal brain tissues (3). Patients with advanced glioblastoma commonly present with seizures and/or stroke, which increases the difficulty and risk of clinical treatment (4). Statistical analysis in previously published data revealed that glioblastoma accounts for ~75% of all malignant tumors associated with the brain (5). According to characteristics of pathologic evaluation and infiltrative growth, different malignant grades result in diverse glioblastoma shapes (6). Therefore, developing treatments for glioblastoma has been a focus of research.

The majority of conditional treatment schedules for human cancers remain ineffective and are often toxic for normal cells (7). Targeted therapy has demonstrated beneficial potential as it specifically targets cancer cells and elicits low toxicity to normal human tissues, demonstrating an increased capacity to eradicate human cancer (8-11). Targeted therapies often involve the application of a specific antibody or receptor for tumor molecules, proteins, peptides, or nucleic acids, as well as efforts that use adoptive transfer of effector cells that directly target antigens on tumor cells (12,13). Clinical research has demonstrated that these novel treatments are effective in treating glioblastoma (14,15). The present study evaluated the preclinical outcomes of lenvatinib targeting of receptor tyrosine kinases for glioblastoma therapy, which promoted cytotoxic T lymphocyte (CTL) responses and interferon-γ (IFN-γ) release through stimulating immunization and adoptive transfer of effector cells that directly target glioblastoma cells in xenograft mice. In addition, the present study discussed the advantages and efficacy of lenvatinib and how over the next decade investigators will attempt to broaden the reach, increase the efficacy and simplify the application of lenvatinib.

Lenvatinib is a multi-targeted tyrosine kinase inhibitor that targets fibroblast growth factor receptors 1-4, vascular endothelial growth factor (VEGF) receptors 1-3, ret proto-oncogene, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene...
homolog and platelet-derived growth factor receptor β (16). Previous studies have demonstrated that angiogenesis mediated by these receptors is involved in the tumorigenesis, development and metastasis of glioblastoma (17,18). Lenvatinib has demonstrated significant anticancer potential against the majority of human cancers in clinical trials, mediated by its inhibition of angiogenesis (19,20). In addition, several reports have revealed that inhibition of the VEGF pathway presented beneficial clinical outcomes in cancer therapy, indicating that targeted therapy using lenvatinib for the treatment of differentiated thyroid cancer and renal cell carcinoma has significant efficacy (16,19). Furthermore, as well as the clinical benefits presented by lenvatinib treatment alone for human cancer therapy, previous studies have also demonstrated that lenvatinib combined with everolimus extended overall survival significantly compared with everolimus alone in patients with metastatic renal cell cancer (20,21). However, few studies have reported the therapeutic effects of lenvatinib regarding the treatment of glioblastoma.

Glioblastoma therapy has attracted scientists and scholars to research more effective and comprehensive treatments (22,23). In the present study, the therapeutic effects of lenvatinib were investigated in a glioblastoma mouse model. The results indicated that targeted therapy of lenvatinib significantly suppressed tumor growth and prolonged the survival of tumor-bearing mice. These results supported a clinical application for lenvatinib in patients with glioblastoma.

Materials and methods

Ethics statement. All animal experiments were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The operation was approved by Chinese Association for Laboratory Animal Sciences, Animal Health Products and the Committee on the Ethics of Animal Experiments Defense Research. All surgery and euthanasia were performed under anesthesia with sodium pentobarbital (50 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All efforts were made to minimize the suffering of the experimental mice.

Cell culture and reagents. BV-2, C6, BC3H1 and G422 glioma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The BV-2 and C6 cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker; Lonza Group, Basel, Switzerland), 50 µg/ml gentamicin (BioWhittaker; Lonza Group), 3 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). BC3H1 and G422 cells were cultured in Eagle's minimum essential medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS.

Apoptosis assays following lenvatinib treatment. BV-2, C6, BC3H1 and G422 (1x10³) cells were incubated with lenvatinib in 96-well plates for 48 h in triplicate for each condition, and PBS was added instead of lenvatinib as a control. BV-2, C6, BC3H1 and G422 cells were grown at 37°C with 5% CO₂ until 80% confluence was reached. Following incubation for 48 h, apoptosis was assessed by incubation of these cells with lenvatinib. The BV-2, C6, BC3H1 and G422 cells were trypsinized and collected into EP tubes. The cells were then washed with cold PBS three times and adjusted to a concentration of 1x10⁶ cells/ml with PBS. The cells were subsequently labeled with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Annexin V-FITC kit; BD Biosciences, San Jose, CA, USA) and analyzed with a FACScan flow cytometer using WinMDI software (version 2.9; BD Biosciences).

Flow cytometry analysis. Tumors from experimental mice were ground into monoplast suspensions and washed three times with PBS for flow cytometric analysis. Tumor cell suspensions were filtered through a 100 µm nylon strainer and centrifuged (1,000 x g for 5 min at 4°C) to remove cell debris. The tumor cells were subsequently incubated with CD3 and CD45-labeled CD4 and CD8 cell subsets in the total infiltrated immune cells. The stained cells were analyzed using a Becton Dickinson FACScan flow cytometer using WinMDI software (version 2.9; BD Biosciences).

IFN-γ release and CTL response assays. Splenocytes were obtained from the euthanized mice, which had been treated by lenvatinib or PBS. Splenocytes were subsequently isolated by passing the spleens through 100 µm nylon mesh filters. The cells were washed three times with PBS and incubated with mitomycin-inactivated BV-2 cells (mitomycin-C, Cat. no. ab120797; Abcam, Cambridge, UK), and IFN-γ was measured in the supernatants on day 3 using a sandwich ELISA kit (Cat. no. ab193969; Abcam). In addition, T cells (1x10⁶) from the splenocytes were purified, as previously described (24) and co-cultured with fresh BV-2 cells for 4 h at the effector:target ratios of 5:1, 15:1 and 45:1. Specific CTL responses to the target cells (BV-2) were analyzed using MTT cytotoxicity assays.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total mRNA was isolated using an mRNeasy Extraction kit (Qiagen, Inc., Valencia, CA, USA). Extracted mRNA (1 µg) was transcribed into cDNA using a reverse transcription kit (Qiagen, Inc.). The cDNA (10 ng) was used for qPCR using the SYBR Green Master Mix system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 30 cycles. All the forward and reverse primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table 1). PCR amplification followed preliminary denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing at 64°C for 30 sec, and 72°C for 10 min. The reaction was performed at a volume of 20 µl, containing 50 ng genomic DNA, 200 µM dNTP, 2.5 units Taq DNA polymerase and 200 µM primers. Relative mRNA expression changes were calculated by 2^-ΔΔCt (25). The results were expressed as n-fold of the expression of β-actin.

Animal experiments. Male BALC/c nude mice (n=100; age, 6-8 weeks; 30-35 g) were purchased from the West China Experimental Animal Center of Sichuan University (Chengdu, China). All animals were housed in a temperature-controlled environment.
Table I. Sequences of primers were used in the present study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| Caspase-3 | F: 5'-AAAGTTTTTCAATGACCAAGC-3'  
|           | R: 5'-TCTGAGAATCTCCTCACAC-3' |
| Caspase-8 | F: 5'-AGTCTATTTTTATATGGGCTCG-3'  
|           | R: 5'-TGGATGTTTATGGTCACCTTTTC-3' |
| Caspase-9 | F: 5'-ATGGGAAACACTGAAAAACTC-3'  
|           | R: 5'-TGTGAGCATGGAAACAATAAC-3' |
| Caspase-10| F: 5'-CTTATCTATGGGACAGACGGGC-3'  
|           | R: 5'-GTGCTCCATTTTTCACAGGGTCCGA-3' |
| β-actin   | F: 5'-AGCCTTCTCCATGGTCGTA-3'  
|           | R: 5'-CGGAGTCAACGGATTGGTC-3' |

Table II. Treatment-related adverse events of lenvatinib with an overall incidence ≥10%.

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>Total (n=50)</th>
<th>0.08-0.16 mg (n=20)</th>
<th>0.24 mg (n=10)</th>
<th>0.32-0.40 mg (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Nausea</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Vomiting</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lethargy</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Fatigue</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Constipation</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>2</td>
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</table>

Lenvatinib effectively induces apoptosis glioblastoma in vitro. To confirm the efficacy of lenvatinib on glioma cells, BV-2, C6, BC3H1 and G422 cells were used to detect the apoptosis rate in vitro induced by lenvatinib (0.24 mg/ml). The apoptosis rate of BV-2 (Fig. 1A), C6 (Fig. 1B), BC3H1 (Fig. 1C) and G422 cells (Fig. 1D) was significantly upregulated following treatment with lenvatinib compared with the control group. In addition, apoptosis-associated gene expression was analyzed in lenvatinib-treated BV-2, C6, BC3H1 and G422 cell lines in vitro. The mRNA expression levels of pro-apoptotic genes were analyzed using RT-qPCR, including caspase-3, -8, -9 and -10. Expression levels of caspase-3 (Fig. 2A), caspase-8 (Fig. 2B), caspase-9 (Fig. 2C) and caspase-10 (Fig. 2D) were significantly increased following 48 h treatment with lenvatinib compared with the control group. These results suggested that lenvatinib-induced apoptosis and upregulated pro-apoptotic genes inBV-2, C6, BC3H1 and G422 glioma cell lines.

Lenvatinib significantly inhibited tumor growth in a glioblastoma mouse model. Following the in vitro apoptosis assays, the present study investigated whether lenvatinib-treated mice demonstrated tumor growth inhibition. BV-2 cells were subcutaneously injected into BALB/c nude mice. Lenvatinib treatment via intravenous injection was initiated when the tumor diameter reached 5-6 mm on day 7 following the first tumor inoculation. The treatment was continued for 14 day cycles at frequency of once daily at the MTD dose (0.24 mg/day). The tumor diameter was recorded and tumor volume was calculated. Tumor growth was significantly inhibited in lenvatinib-treated mice (Fig. 3A). In addition, lenvatinib prolonged the survival of tumor-bearing mice across 120 days of observation compared with control group (Fig. 3B). These results suggested that the therapeutic effects of lenvatinib on the BV-2-bearing mice were strong enough to suppress the glioma tumor growth, which translated into long-term survival.
Treatment with lenvatinib resulted in immunological cytotoxicity and a CTL response in glioblastoma tumors. Tumor-bearing mice from the lenvatinib and PBS-treated groups (n=8 mice/group) were sacrificed on day 30 to further analysis of beneficial outcomes. Tumors were collected on day 30 in each group. The tumors were subsequently ground, filtered and stained for CD4+ and CD8+ expression on tumors. Tumors from the mice treated with lenvatinib exhibited a significantly increased degree of CD4+ (Fig. 4A) and CD8+ (Fig. 4B) cell infiltration in the glioblastoma tumor model, as determined by
Student's paired t-tests. In addition, CTL responses against the BV-2 glioblastoma cells were assessed on day 30. BV-2-specific CTL activity was assessed following the purification of T cells co-cultured with tumor cells. Treatment with lenvatinib resulted in a significant promotion of CTL activity compared with the PBS-treated group at the effector:target ratios of 15:1 and 45:1 (Fig. 4C). Furthermore, IFN-γ release assay was analyzed to identify and explain the long-term survival in lenvatinib-treated mice. Lenvatinib treatment resulted in significantly increased IFN-γ release compared with PBS-treated mice (Fig. 4D).

These results suggested that treatment of tumor-bearing mice with lenvatinib resulted in the generation of tumor-specific CTL responses and partial protection of the animals against the tumor cells, which may contribute to the long-lasting antitumor effects observed in the BV-2-bearing mice.

Discussion

Targeted therapy has demonstrated marked antitumor activities in the clinical treatment of several types of human cancer (16,28,29). Antineoplastic agents with targeted therapy agents can effectively target tumor cell-specific recognition domains, either antigens or receptors (30-33). Different targeted therapy drugs for cancer treatment are being studied clinically. The targeted therapy agents frequently use tumor cell-specific recognition to inhibit tumor angiogenesis, mediated by suppression of tumor-derived vascular endothelial cells, and have demonstrated encouraging results in the treatment of certain advanced tumors (34,35). However, more targeted therapy agents require development to cater to the needs of an ever-increasing number of cancer patients.
Therapeutic protocols that target VEGF-mediated pathways have been clinically applied to treat human cancers (36). However, single therapies that target VEGF remained challenges in clinical outcomes, and improvements are required to overcome ineffective drug treatments (37). Lenvatinib is a multi-targeted tyrosine kinase inhibitor with anticancer potential, which improved upon and overcame deficiencies of single anticancer agents, as well as demonstrated improved therapeutic benefits for patients with cancer (38,39). However, few studies among these previous reports studied the efficacy of lenvatinib as a therapy for glioblastoma. In the present study, an optimal treatment scheme of lenvatinib for glioblastoma in a murine model has been defined, and preclinical efficacy has demonstrated low toxicity and positive outcomes. The data support the use of the effective, multi-targeted lenvatinib agent for glioblastoma therapy, as it is associated with improved efficacy of treatment and manageable, lower toxicity compared with other anticancer agents (40).

Lenvatinib has previously demonstrated favorable anti-tumor potential for human cancer therapy (37). Lenvatinib is also well tolerated without serious treatment-associated adverse events. Despite targeted therapy providing the advantage of tumor specificity, it is conceivable that effectively invoked toxicity of immune cells for tumor cells may contribute to cancer therapy (40). The clinical application of lenvatinib would be more effective by intravenous injection to inhibit tumor cell growth and produce tumor-specific killer cells with improved immunogenicity to stimulate adaptive T cell mediated anti-tumor immunity (41).

Although previous studies have demonstrated the MTD of lenvatinib in phase II trial in advanced medullary thyroid cancer (36). In the present study, targeted therapy was introduced to treat glioblastoma in BV-2-bearing BALB/c nude mice. In addition, lenvatinib demonstrated manageable toxicity at the MTD, and lower-dose cohort and MTD dose presented an effective treatment for glioblastoma. Furthermore, the treatment-associated adverse events of lenvatinib treatment were representative and consistent with previous clinical research (41).

The responses observed with the present lenvatinib therapy conferred an advantage relative to other single-target therapy (42). In the present primary analysis of glioblastoma therapy, treatment with 0.24 mg lenvatinib once daily resulted in an increased CTL response, increased IFN-γ release and improved long-term survival in glioblastoma-bearing mice.

In conclusion, a novel therapeutic schedule for the treatment of glioblastoma using the multi-therapy anticancer agent lenvatinib was introduced as an MTD dose of 0.24 mg at a frequency of once daily. The treatment-associated adverse events were consistent with those of multi-targeted tyrosine kinase inhibitors and were managed effectively by administering continuously in 14 day cycles. However, the beneficial effects of targeted lenvatinib therapy require further elucidation to improve the clinical value of this regimen.

References


