Preclinical anti-angiogenic and anti-cancer activities of BAY1143269 in glioblastoma via targeting oncogenic protein expression

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Abstract
Glioblastoma angiogenesis is critical for tumor growth, making it an appealing target for treatment development. BAY1143269 is a novel inhibitor of mitogen-activated protein kinase interacting serine/threonine-protein kinase 1 (MKN1) and has potent anti-cancer activity. We identified BAY1143269 as an angiogenesis inhibitor, by in vitro and in vivo glioblastoma angiogenesis models. BAY1143269 inhibited the capillary network formation of glioblastoma microvascular endothelial cells (GMECs), particularly the early stage of tubular structure formation. It also inhibited migration and proliferation, and induced apoptosis of GMECs isolated from glioblastoma patients. We found that BAY1143269 acted on GMECs by suppressing the eukaryotic translation initiation factor 4E (eIF4E) and eIF4E-mediated expression of oncogenic proteins, including those involved in cell cycle, epithelial-mesenchymal transition (EMT), and pro-survival. In addition, BAY1143269 suppressed eIF4E phosphorylation, inhibited proliferation, and induced apoptosis of glioblastoma cells. Interestingly, it reduced vascular endothelial growth factor (VEGF) level in tumor cells and culturing medium, demonstrating the inhibitory effect of BAY1143269 on tumor proangiogenic microenvironment. We finally challenged BAY1143269 on the glioblastoma xenograft mice model and observed a significant tumor growth reduction without toxicity in mice receiving oral BAY1143269. Immunoblotting analysis demonstrated significantly less phosphorylated-eIF4E (p-eIF4E), cluster of differentiation 31 (CD31) (microvascular endothelial cell marker), and VEGF in tumors from drug-treated mice. In summary, the inhibition of glioblastoma angiogenesis with BAY1143269 may provide an alternative approach for anti-glioblastoma therapy.

KEYWORDS
BAY1143269, eIF4E, glioblastoma angiogenesis, VEGF

Abbreviations: CD31, cluster of differentiation 31; eIF4E, eukaryotic translation initiation factor 4E; EMT, epithelial-mesenchymal transition; GMECs, glioblastoma microvascular endothelial cells; MKN1, mitogen-activated protein kinase interacting serine/threonine-protein kinase 1; p-eIF4E, phosphorylated-eIF4E; VEGF, vascular endothelial growth factor.
1 | INTRODUCTION

Glioblastoma is the most common and aggressive form of brain cancer with average overall survival of approximately 1 year. Since the introduction of adjuvant temozolomide, the gold standard therapy for glioblastoma has remained unchanged but the prognosis has improved little over the past few decades. Further research is warranted into developing therapeutic strategies to advance the glioblastoma management. Glioblastoma is characterized with intra- and intertumor heterogeneity, enriched stem cancer cell, and extensive vascularization. Vascularization, the most important feature of glioblastoma, is associated with prognosis: the higher degree of angiogenesis is significantly correlated with shorter progression-free survival. Angiogenesis is the formation of blood vessel from pre-existing ones. Vascular endothelial growth factor (VEGF), the most important and specific angiogenesis growth factor, plays a vital role in promoting tumor angiogenesis and is highly expressed in tumor cells. Bevacizumab, an angiogenesis inhibitor, has entered phase III clinical trial to treat recurrent glioblastoma in combination with reirradiation and immunotherapy.

The mitogen-activated protein kinase (MAPK)-interacting kinase (MNK) activity is important for the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) at Ser209, which preferentially promotes the synthesis of pro-angiogenic, anti-apoptotic, oncogenic proteins that favor tumor growth and survival, such as myeloid cell leukemia 1 (MCL1), VEGF, Snail, and Cyclin D1. Thus, MNK has been the target of major drug discovery programs and MNK inhibitors have been hotly evaluated in clinical trials for cancer treatment in recent years. BAY1143269 is a potent and selective novel small-molecule inhibitor of mitogen-activated protein kinase interacting serine/threonine-protein kinase 1 (MKN1). BAY1143269 regulates downstream factors involved in cell cycle regulation, apoptosis, and epithelial–mesenchymal transition (EMT) and has potent efficacy in patient-derived non-small-cell lung cancer xenograft models. In this study, we investigated the effects of BAY1143269 on glioblastoma angiogenesis and growth in multiple disease models, and attempted to analyze the underlying mechanisms of BAY1143269’s action in glioblastoma.

2 | MATERIALS AND METHODS

2.1 | Glioblastoma cell lines and drugs

Human glioblastoma cell lines T98G and U373 used in our study were authenticated using short tandem repeat (STR) profiling analysis (Precision Gene Biotechnology Inc.) and examined for mycoplasma contamination (Thermo Fisher) prior to experiments. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Pen/Strep) under 5% CO₂ at 37°C. BAY1143269 (DC Chemicals, Cat. No.: DC20750) was reconstituted in dimethyl sulfoxide (DMSO).

2.2 | Patient tissues and glioblastoma microvascular endothelial cells (GMECs) isolation

Glioblastoma tissues were obtained during surgery from patients seen at the Affiliated Hospital of Southwest Medical University after signed informed consent as approved by the Southwest Medical University Institutional Review Board. GMECs were enriched from glioblastoma tissues and purified using fluorescence-activated cell sorting (FACS) using the same protocol, as described in our previous study. GMECs were cultured in endothelial cell medium (Cell Systems, Cat No. 420-500).

2.3 | Proliferation and apoptosis assays

For cell proliferation, cells were plated at 1×10⁴ cells per well in triplicate in a 24-well plate, and cell proliferation was determined at 3 days after drug treatment using 5-bromo-2′-deoxyuridine (5-BrdU) cell proliferation assay (Millipore) with absorbance reading at 490 nm.

For cell apoptosis, cells were plated at 1×10⁶ cells per well in triplicate in a 6-well plate, and apoptosis was determined at 3 days after drug treatment using Cell Death Detection ELISA (Roche) with absorbance reading at 405 nm. Time-course analysis of apoptosis was performed after 6, 24, 48, and 72 h drug treatment.

2.4 | Migration assay

Cell migration assay was performed using Corning chambers with Matrigel following the manufacturer’s protocol. Briefly, endothelial cells (ECs) suspended in serum-free medium together with drug were added into top chambers, while medium containing 10% FBS was placed in the bottom chambers as a chemoattractant. After 8 h, non-migrated cells on the inner membrane were gently removed by wiping with a cotton swab. The migrated cells at the bottom surface of the membrane were fixed with 4% paraformaldehyde. Cells were washed with phosphate-buffered saline (PBS) and stained with 0.1% crystal violet. Cells were photographed and counted under microscope.

2.5 | Capillary network formation assay

Capillary network formation assay was performed using Corning Matrigel matrix. One hundred fifty microliters of Matrigel matrix was added to the 96-well plate and rapidly polymerized at 37°C. Fifty microliters of a mixture of cells, drug, and medium was plated onto the top of Matrigel. After 6 h incubation, a tube-like structure was formed and the images were taken under an inverted microscope. ImageJ software was used to quantify the length of branches of the whole well.
2.6 | Western blotting

After 24 h drug treatment, cell lysates were prepared in radioimmunoprecipitation assay buffer (RIPA) which is a lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare). Membranes were probed with the designated primary antibodies (1:1000, Cell Signaling) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Cell Signaling). The protein bands were visualized using chemiluminescence (enhanced chemiluminescence (ECL), GE Healthcare) and the intensity of the bands was quantified using ImageJ software.

2.7 | Measurement of supernatant levels of VEGF and PDGF

As many as $10^6$ cells were seeded onto a 6-well plate and treated with drug for 24 h followed by supernatant collection. The supernatant levels of VEGF and PDGF were measured using human VEGF and PDGF ELISA Kit (Thermo Fisher Scientific) as per manufacturers' protocol.

2.8 | Glioblastoma xenograft growth in vivo

All animal experiments were conducted in vivarium of Southwest Medical University in accordance with the guidelines required by the Institutional Animal Care and Use Committee. The 4- to 6-week-old male SCID (severe combined immunodeficient) mice were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd., housed in a specific pathogen-free vivarium, and fed standard chow. Glioblastoma xenograft mouse model was established using T98G cells as previously described in our study. After the development of palpable tumors, mice were randomly divided into two groups (n = 5 per group) for vehicle control or daily oral 100 mg/kg BAY1143269 treatment. After 25 days, mice were euthanized with CO$_2$ inhalation. Tumors were isolated and lysed for total protein extraction.

2.9 | Statistical analyses

In vitro assays were performed at least three times with each sample duplicate or triplicate. Comparisons between two groups were performed using Student’s t-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) test. Each datapoint shows the mean detected value from five different mice. A p-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | BAY1143269 inhibits early stage of glioblastoma angiogenesis via suppression of GMECs morphogenesis, migration, growth, and survival

To determine whether BAY1143269 has an effect on angiogenesis, we performed Matrigel tube formation assay, a well-established in vitro angiogenesis assay that is based on the differentiation of endothelial cells and the formation of tube-like structures on an extracellular matrix. To mimic a clinically relevant glioblastoma angiogenesis model, we used microvascular endothelial cells (GMECs) isolated from glioblastoma tissues from patients. We observed an extensive tubule formation within 6 h of plating GMECs onto Matrigel in control (Figure 1A). However, GMECs exposed to BAY1143269 hardly formed tubular structures. Quantification of Matrigel tube formation showed that BAY1143269 inhibited GMECs tubular structure formation in a concentration-dependent manner (Figure 1B).

Time-course analysis of Matrigel tube formation assay was performed by adding BAY1143269 at 0, 1, and 3 h after plating GMECs to Matrigel. Concurrent addition (0 h) of BAY1143269 and GMECs to Matrigel demonstrated the most inhibitory effect on tubular structure formation and there was a gradual loss of inhibition when BAY1143269 was added at later time points (1 and 3 h). In addition, we showed that BAY1143269 inhibited GMECs migration and proliferation (Figure 2A–C and Figure S1A,B). BAY1143269 also increased apoptosis in GMECs as assessed by the quantification of DNA fragmentation (Figure 2D and Figure S1C). Time-course analysis of apoptosis demonstrated that BAY1143269 initialized apoptosis after 24 h treatment (Figure 2E and Figure S1D). In addition, 6 h treatment of BAY1143229 did not induce significant apoptosis in GMECs. This result excludes the possibility that the disrupted formation of tubular structure by BAY1143269 is a consequence of GMECs apoptosis.

3.2 | BAY1143269 inhibits elf4E, EMT, cell cycle, and pro-survival factors in GMECs

To understand the molecular mechanisms of BAY1143269’s action in GMECs, we first investigated elf4E-mediated signaling, as elf4E is the major substrate of MNK1. Immunoblotting analysis of GMECs exposed to BAY1143269 displayed decreased level of phosphorylated elf4E (p-elf4E) but not total elf4E (Figure 3A,D). As expected, the levels of Cyclin D1 and c-Myc, downstream targets of elf4E and cell cycle factors, were decreased by BAY1143269 (Figure 3A,B). We observed reduced expression of other cell cycle factors, including Cyclin A2 and G0/G1-associated cyclin-dependent kinase (CDK)-2, in GMECs exposed to BAY1143269. In addition, the level of EMT factors, such as Snail, Slug, and zinc finger E-box-binding homeobox 1 (ZEB1), was decreased by BAY1143269 (Figure 3A,C). The level of Bim was increased
and the level of Mcl-1 was decreased in BAY1143269-treated GMECs (Figure 3A,E). Taken together, our results demonstrate that BAY1143269 inhibits elf4E, suppresses EMT, decreased cell cycle and pro-survival factors, and increased anti-pro-apoptotic factor in GMECs.

3.3 | BAY1143269 inhibits growth, survival, elf4E, and VEGF in glioblastoma cells

To understand whether BAY1143269 has an effect on glioblastoma, we treated glioblastoma cells with BAY1143269 and then measured proliferation and apoptosis. Similar to GMECs, BAY1143269 concentration-dependently decreased proliferation and increased apoptosis in both T98G and U373 cell lines (Figure 4A,B). In addition, BAY1143269 decreased the level of p-elf4E without affecting elf4E (Figure 4C). Consistent with the finding that elf4E facilitates cap-dependent translation of angiogenesis-promoting messenger RNAs (mRNAs) such as VEGF, BAY1143269 treatment resulted in the reduction of VEGF in T98G and U373 cells. BAY1143269 did not affect the PDGF level in glioblastoma cells. We further measured the levels of VEGF and PDGF secreted in the supernatants of glioblastoma cells. We found that there was significantly less VEGF but not PDGF in the supernatants of BAY1143269-treated T98G and U373 cells (Figure 4D). Collectively, BAY1143269 inhibits growth, survival, elf4E, and VEGF in glioblastoma cells.

3.4 | BAY1143269 inhibits glioblastoma angiogenesis and growth, and decreases secretion of VEGF in mice

We further challenged BAY1143269 on glioblastoma xenograft mouse model to determine whether BAY1143269 inhibits
glioblastoma angiogenesis and growth in vivo. We established glioblastoma xenograft model using the same method described in our previous study. Mice with palpable tumor were randomly divided into two groups: vehicle control and 100 mg/kg BAY1143269 given by oral gavage. Compared to control mice group, we did not observe any reduction in body weight (Figure 5A) or abnormal appearance/behavior in mice receiving drug treatment, indicating that BAY1143269 at 100 mg/kg is not toxic to mice. However, we observed a significant reduction in tumor size in mice receiving BAY1143269 (Figure 5B). Cluster of differentiation 31 (CD31) is expressed on all cells within the vascular compartment and is a classical marker to identify and label microvascular vessels.

In line with our in vitro data, immunoblotting analysis of CD31 molecule showed consistently less CD31 level in tumor from BAY1143269-treated mice than control mice (Figure 5C and D). In addition, we observed significantly reduced VEGF but not PDGF levels in tumor from BAY1143269-treated mice. These results demonstrate that BAY1143269 inhibits glioblastoma angiogenesis and growth, and decreases secretion of VEGF in mice.

4 | DISCUSSION

Consistent with the fact that glioblastoma is a highly vascular tumor, previous studies of ours and others demonstrate that angiogenesis inhibition is effective in inhibiting glioblastoma growth. Efforts of our lab and others to explore alternative therapeutic strategy for glioblastoma treatment had focused on the compounds that target microvascular endothelial cells. The current findings extend the repertoire of therapeutic strategy to include the compounds that target microvascular endothelial cells, tumor cells, and microenvironment. This study provided strong evidence to show that BAY1143269 (1) inhibits in vitro and in vivo glioblastoma angiogenesis; (2) suppresses proliferation and induces apoptosis in glioblastoma cells; and (3)
decreases the level of VEGF, a proangiogenic cytokine, in glioblastoma tissues.

As a MNK1 inhibitor, the anti-cancer activities of BAY1143269 have been well established.\cite{10} However, the effect of BAY1143269 on tumor angiogenesis is unknown. In line with the studies that other MKN inhibitors, such as cercosporamide, have anti-angiogenic activity,\cite{18,19} our work demonstrated that BAY1143269 inhibited glioblastoma angiogenesis by suppressing GMECs morphological differentiation, migration, growth, and survival. Using the glioblastoma xenograft mouse model, we further showed that BAY1143269 inhibited glioblastoma angiogenesis in mice as shown by the decreased CD31 level. In our study, the levels of CD31, VEGF, and PDGF were analyzed at the endpoint of tumor progression. Time-course analysis would be performed to monitor angiogenesis in control and BAY1143269-treated tumors. Compared to studies that investigate inhibitors of tumor angiogenesis using endothelial cells (ECs) isolated from normal tissues,\cite{20,21} microvascular endothelial cells used in our study are GMECs that are isolated and further purified from glioblastoma tissues from various patients, which are specific to glioblastoma angiogenesis and represent tumor angiogenesis. BAY1143269 is also active against glioblastoma cells by suppressing proliferation and inducing apoptosis. The dual inhibition of BAY1143269 on angiogenesis and tumor gives it more advantages than angiogenesis inhibitors for the treatment of glioblastoma.

As an oncogene that mediates the translation of tumor favorable proteins, eIF4E is an interest for drug development for many years but has been very challenging to develop small molecules to target due to the nature of its binding site.\cite{15} BAY1143269 potently decreased phosphorylation of eIF4E and suppressed levels of subsequent downstream targets of eIF4E. The proteins decreased by BAY1143269 in GMECs and glioblastoma cells are those that critically promote cell cycle, EMT, survival, and angiogenesis. The mechanisms of BAY1143269’s action in our study are supported by the previous studies that MNK/eIF4E inhibition leads to the suppression of many oncogenic proteins.\cite{22,23} Importantly, eIF4E phosphorylation at Ser209 by Mnk is only required in tumor but not normal cells,\cite{24} making Mnk as a safe target for the treatment of cancers. This is consistent with our findings that BAY1143269 inhibits glioblastoma angiogenesis and growth without causing significant toxicity in mice. VEGF is the most important angiogenesis growth

**FIGURE 3** BAY1143269 inhibits eukaryotic translation initiator factor 4E (eIF4E)-mediated signaling pathways and epithelial-mesenchymal transition (EMT) signaling in glioblastoma microvascular endothelial cell (GMECs). Western blotting (A) and quantification (B–E) for phosphorylated-eIF4E (p-eIF4E), c-Myc, Cyclin A2, Cyclin D3, cyclin-dependent kinase (CDK)-2, Snail, Slug, zinc finger E-box-binding homeobox 1 (ZEB1), Bim, and Mcl-1 in GMECs exposed to BAY1143269 for 24h. Molecules were normalized to β-actin. *, p < .05, compared to control.
The ability of BAY1143269 in decreasing the VEGF level in tumor cells, supernatant of cell culturing medium, and tumor tissues correlates well with its potent anti-angiogenic activity in the glioblastoma xenograft mouse model.

Although bevacizumab is in clinic use for the treatment of adults with recurrent glioblastoma based on the improvement in progression-free survival and additional palliative benefits, phase III studies have demonstrated no extension of overall survival. Among 33 active clinical trials, 4 phase III trials are recruiting to investigate whether bevacizumab in combination with irradiation, chemotherapy, or immunotherapy can convey a survival benefit (ClinicalTrials.gov. NCT02761070, NCT05118776, NCT05271240, and NCT04277221). As bevacizumab is a monoclonal antibody, other small-molecule anti-angiogenic inhibitors, such as sorafenib, sunitinib, and pazopanib, might have advantage than bevacizumab to target glioblastoma. The ability of BAY1143269 in targeting VEGF as well as other oncogenic proteins suggests that BAY1143269 may circumvent the problem of acquired resistance to bevacizumab. It is worthy of comparing the inhibitory effects of BAY1143269 with those of bevacizumab as well as other small-molecule angiogenesis inhibitors.

The ability of compounds in penetrating blood-brain barrier is important for the development of anti-glioblastoma drugs. We noted that the in vivo glioblastoma model used in our study is established by subcutaneous implantation. Future work to evaluate the ability of BAY1143269 to cross the blood-brain barrier is desirable. Glioblastoma xenograft transplantation model using cell lines or patient-derived xenograft model using primary glioblastoma cells should be applied.

In summary, our findings systematically demonstrate the anti-angiogenic and anti-cancer activities of BAY1143269 in glioblastoma.
Our work also highlights the therapeutic value of targeting MNK/eIF4E in glioblastoma, and possible other cancers that are highly vascularized.

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**DISCLOSURE**
All authors declare no conflict of interest.

**ETHICS STATEMENT**
The procedures with animal work were approved by the Institutional Animal Care and Use Committees of Southwest Medical University and were conducted in accordance with the recommendations.

**AUTHOR CONTRIBUTIONS**
Weifeng Wan and Xin Zhang performed the experiments, collated the results and wrote the manuscript; Changren Huang and Ligang Chen performed the experiments; Xiaobo Yang and Kunyang Bao analyzed and consolidated the data; Tangming Peng analysed the results and supervised the project. All approved the final manuscript.

**DATA AVAILABILITY STATEMENT**
Datasets that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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