The Antitumor Activity and Mechanism of MCL3 in G422 Glioblastoma

Qian-Qian Du, Mei Tang, Lu-Lu Huang, Ru Zhao, Chen Yan, Yan Li, Xian-Dao Pan

Objective: Parthenolide (PTL) induces anti-tumor effects via the nuclear factor kappa B (NF-kB) signaling pathway. MCL3, a PTL derivative, is a sesquiterpene lactone synthesized by the rearrangement and subsequent oxidation of PTL. The aim of this study was to elucidate the antitumor activity and mechanism of action of MCL3 in glioblastoma (GBM). Materials and Methods: The effects of MCL3 on G422 cell proliferation, apoptosis, invasion, and angiogenesis in vitro were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry, the cell invasion, and tube formation assays. The subcutaneously transplanted G422 xenograft model was used to detect the effect of MCL3 on tumor growth in vivo. Pathological changes were analyzed by immunohistochemical staining. The effects of MCL3 on NF-kB and Stat3 transcriptional activities were examined using a dual-luciferase reporter assay. Protein levels related to the NF-kB/interleukin (IL)-6/Stat3 signaling pathway were determined using western blot analysis. Results: MCL3 inhibited GBM cell proliferation, invasion, and angiogenesis in a concentration-dependent manner. Moreover, MCL3 decreased the transcriptional activities of NF-kB and Stat3. MCL3 suppressed tumor growth in the subcutaneously transplanted G422 xenograft model, while the inhibition rate was 79% in tumor weight at 40.0 mg/kg. MCL3 blocked the NF-kB/IL-6/Stat3 signaling pathway in G422 cells and tumors, resulting in the downregulation of Stat3 target genes related to apoptosis, invasion, etc. Conclusion: The results show that MCL3 might inhibit G422 GBM growth partly due to the inhibition of the NF-kB/IL-6/Stat3 signaling pathway.

Keywords: Glioblastoma, MCL3, nuclear factor kappa B/interleukin-6/Stat3, parthenolide derivative

INTRODUCTION

Glioblastoma (GBM) is the most frequent and aggressive primary malignant brain tumor related to poor prognosis and limited treatment.[1] Adjuvant radiotherapy combined with chemotherapy is typically prescribed for patients, although some patients undergoing this treatment also had increased tumor size and side effects. Therefore, it is important to improve the treatment options for patients with GBM.[2,3]

Traditional Chinese Medicine (TCM) has formed its own unique system of theories, diagnostics and therapies in China, and studies have shown that TCM could play an important role in cancer prevention and treatment.[4] Many countries, including the United States of America, have attempted to undertake TCM-related research projects.[5] TCM, in combination with chemotherapy, could suppress tumor growth, decrease their side effects.[4,6] In the last few decades, TCM has been used widely for cancer therapy around the world.

Sesquiterpene lactones including parthenolide [PTL, Figure 1] were extracted from the feverfew plant (Tanacetum parthenium), which were primarily used for the treatment of skin infection, arthritis, and migraines.[6] In recent studies, PTL was...
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In this study, the observed to inhibit proliferation of cancer cells, particularly prostate, breast, gastric, renal cell carcinoma, lung, and colon cancers. Moreover, there is evidence that PTL exhibits anti-tumor activity, including against GBM, by suppressing the activation of nuclear factor kappa B (NF-κB) mediated by 1xK, resulting in inhibition of interleukin (IL)-6-induced Stat3 activation. This mechanism may be the Michael addition reaction of a thiol group on Cys38 of p65/NF-κB with PTL. Since NF-κB was associated with tumor invasion, metastasis, and drug resistance, suppression of NF-κB could improve the sensitivity of cancer cells to anti-cancer drugs.

MCL3 [Patent: 201711285215.2, Figure 1] was synthesized using a three-step reaction from PTL. In this study, the antitumor activity and mechanism of MCL3 against GBM were investigated in vitro and in vivo. We found that MCL3 induced antitumor activity in G422 GBM by targeting the NF-κB/IL-6/Stat3 signaling pathway.

**Materials and Methods**

**Cell lines and culture**

NCI-H1650 and NCI-H460 human lung cancer cell lines, MCF7 and MDA-MB-231 human breast cancer cell lines, BxPC3 human pancreatic cancer cell line, U-87 MG, U251, and Hs683 human brain tumor cells, HCT-8 human colon cancer cell lines, and BGC-823 human gastric cancer cell line, were obtained from the Cell Center of CAMS and PUMC in Beijing. MGC-803 and HGC27 human gastric cancer cell lines, HCT15 human colon cancer cell lines, Mia-PaCa2 human pancreatic cancer cell lines were received from Tsinghua University in Beijing. Caki-1 and ACHN human renal cell carcinoma cell lines were received from DUKE-NUS Medical School Singapore. Capan2 human pancreatic cancer cell line was obtained from Shanghai XiangF Bio in Shanghai. G422-GFP mouse brain tumor cell line was obtained from Guangzhou Jennio Biotechin Guangzhou. U-87 MG cells were incubated in Minimum Essential Media (MEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) added to 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/mL penicillin, and 100 µg/mL streptomycin. HGC27, HCT15, HCT-8, NCI-H1650, and NCI-H460 were cultured in 1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) added to 10% FBS, 100 IU/mL penicillin, and 10 µg/mL streptomycin. Other cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.,) added to 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

**Drugs and compounds**

MCL3 was synthesized, while Pan and temozolomide (TMZ) were purchased from Shanghai Biolang Bio-tech Co., Ltd. in Shanghai. For the in vitro experiments, MCL3 was made in solution dimethyl sulfoxide (DMSO) solution and maintained at 4°C. DMSO was used as the vehicle control in vitro experiments at a concentration of 0.1%. Considering the experiments in vivo, MCL3 and TMZ were dissolved in a solution of 25% PEG400.

**The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay**

Cells (100 µL) were added to 96-well plates and incubated for 24 h. Different concentrations of MCL3 were then added to per well for 100 µL. After 96 h, 50 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2.0 mg/mL) was added and incubated for a further 4 h. The solution was removed, and 150 µL DMSO was added. The optical density was measured at 570 nm by an ELISA reader (WD-2102A, China). All experiments were performed in triplicate.

**Cell apoptosis assay**

G422 cells were cultured for 24 h, and different concentrations of MCL3 (3.3 mol/L, 10.00 µmol/L, 30.00 µmol/L) were added to per well for 100 µL. Following incubation for 72 h, cells were harvested, and washed with cold PBS. Subsequent to suspension in binding buffer, cells were stained with 100 µL PI. The cell samples were analyzed using ACCURI C6 flow cytometer.

**Cell invasion assay**

An invasion assay was achieved using transwell inserts containing polycarbonate filters with 8.0 µm pores. Matrigel (10 µL, 1.0 mg/mL) was supplied using the upper chamber. The cells (7 × 10⁶ cells/well) were suspended in 200 µL 0.1% BSA with different concentrations of MCL3 (1.11 µmol/L, 3.33 µmol/L, 10.00 µmol/L), and seeded into the upper chamber; 600 µL normal medium (10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin) was placed in the lower chambers. The control group did not add MCL3. Following 24 h incubation at 37°C in an atmosphere of 5% CO₂, the cells on the upper cover of the membrane were mechanically removed, while the invading cells on the lower cover were fixed with methanol, and stained with hematoxylin and eosin (H and E) dye. The number of invasive cells in four random fields per membrane was counted at ×200 magnification (Olympus IX70). The experiment was performed in triplicate.

**Tube formation assay**

A total of 50,000 EAg 926 cells, which were suspended in 100 µL DMEM with different concentrations of MCL3 (3.33 µmol/L, 10.00 µmol/L, 30.00 µmol/L), were added into a 96-well plate precoated with Matrigel. After maintenance at 37°C for 5 h, EA.

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**Figure 1**: Chemical structures of parthenolide, MCL3
hy926 cells were examined for capillary-like tube formation and photographed under a microscope (>100 magnification, Olympus IX70). All experiments were performed in triplicate.

**G422 glioblastoma xenograft model**

Procedures for the animal study were performed with the approval of the Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. Four to 6-week-old male mice (National Institutes for Food and Drug Control) were used. G422 GBM tumors implanted in the axillary of these mice were removed and homogenized. Tumor cells were diluted with aseptic saline in a ratio of 1:3 to form a tumor solution. Test mice were then injected with 0.2 mL tumor solution in the left flank. The tumor-bearing mice were divided into five groups, each containing six mice. All groups were fed with drugs from the next day. The control group was orally fed with 25% PEG400; while the other groups were administered an oral dose of 30.0 mg/kg TMZ for 5 days, followed by 10.0 mg/kg, 20.0 mg/kg, and 40.0 mg/kg MCL3 for 2 weeks. Mice were mercy killed at the final period of the experiment. Tumors were excised, weighted, and photographed. The inhibition rate was calculated based on the size of the tumor and growth period. A sample of the tumor was fixed with 4% paraformaldehyde for immunohistochemical (IHC) staining, while the other tissues were maintained at −80°C to be used in western blot analysis.[18]

### A dual luciferase assay

The luciferase reporter plasmid, including the NF-κB-binding site (pGMNF-κB-Lu), and the TK-Renilla luciferase plasmid (pGMR-TK) were obtained from Genomeditech Shanghai Co. Ltd in Shanghai. G422-GFP cells in 96-well plates were co-transfected with pGMR-TK and pGMGL11-Lu as per the Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) standard protocol. After transfection for 6 h, cells were supplemented with various concentrations of MCL3, and incubated for a further 48 h. Luciferase assays were measured by a dual luciferase reporter assay system (Promega Corporation; cat. no., E1960), as per the product’s protocol. Luciferase values were normalized to Renilla values. The transcription activity of Stat3 was detected by the Eastinno Biotechnology Co. Ltd in Zhejiang by the dual luciferase assay.

### Immunohistochemistry assay

Immunohistochemistry staining for proliferating cell nuclear antigen (PCNA) (1:5000 dilutions) and CD34 (1:5000 dilutions) was performed as previously described.[20-22] Brown stained cells were defined PCNA-positive and CD34-positive cells. The expression of PCNA was quantified using the proliferation index, while CD34 quantified using the microvessel density (MVD) according to the Weidner’s method.[23] MVD is used to analyze the level of angiogenesis. MVD was evaluated by Weidner’s method.[24]

### Western blot assay

Tissues from three randomly selected tumors from G422 xenograft mice of the control, and 20.0 mg/kg and 40.0 mg/kg MCL3 groups were lysed. G422 cells were incubated with MCL3 for 72 h. The total protein in these cells was refined by the radioimmunoprecipitation assay buffer (RIPA, R0020; Beijing Solarbio Science and Technology Co., Ltd) and phenylmethanesulfonyl fluoride (PMSF; P0100; Beijing Solarbio Science and Technology Co., Ltd). Nuclear protein was obtained using the Nuclear and Cytoplasmatic Extraction Kit (CW0199S; CWBIO). The total protein quantity was evaluated using the BCA Protein Assay Kit (PC0020; Beijing Solarbio Science and Technology Co., Ltd). Total proteins (35 μg) and nuclear proteins (25 μg) were maintained separately in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transported to the PVDF membrane. The membranes were blocked using a solution of TBS consisting of 1% Tween® 20 and 5% skimmed dry milk, and maintained with the specific primary antibodies (dilution, 1:1,000; Cell Signaling Technology, Inc., Shanghai, China; Abcam Trading, Shanghai, Ltd) overnight at 4°C. The samples were detected using the secondary antibody of a horseradish peroxidase-labeled goat (dilution, 1:5,000; cat. no., sc-2004; Santa Cruz Biotechnology, Inc.), for 1 h at 25°C an enhanced chemiluminescence western blot system (Applygen Technologies, Inc.,) was used for detection. β-actin and histone H3 were used as loading controls.

### Statistical analysis

Data are expressed as the mean ± standard deviation. Two-tailed independent Student’s t-test (Microsoft Excel, 2010) was performed and P < 0.05 was confirmed to indicate a statistical difference.

### Results

#### MCL3 inhibited the cell proliferation

Anti-proliferation activity was examined in different cancer cell lines treated with MCL3 for 96 h using the MTT assay, with the IC₅₀ values ranging between 6.44 and 23.40 μmol/L [Table 1]. The GBM cell lines were more sensitive to MCL3, including G422, U-87 MG, U251, and Hs683 cell lines. The IC₅₀ values were 8.94 ± 1.33 μmol/L, 6.44 ± 1.80 μmol/L, 14.80 ± 9.30 μmol/L, and 18.90 ± 4.10 μmol/L, respectively [Figure 2].

#### MCL3 induced G422 cell apoptosis

Apoptotic activity of MCL3 was investigated by flow cytometry. After G422 treated with MCL3 for 72 h, the G422 cell apoptotic rate increased in a dose-dependent manner. The apoptotic rates were 20.7 ± 1.4% (P < 0.05) and 29.7 ± 0.8% (P < 0.001) in the 10.00 μmol/L and 30.00 μmol/L groups, respectively. While the control group had a 16.2 ± 1.6% rate of apoptosis [Figure 3]. The result suggests that MCL3 could induce apoptosis in G422 cells.

#### MCL3 suppressed G422 cell invasion

The transwell assay was used to determine the influence of MCL3 on G422 cell invasion ability. MCL3 decreased the number of transmembrane G422 in a concentration-dependent manner. The inhibition rates were...
23.9% \((P < 0.01)\) and 43.6% \((P < 0.01)\) in the 3.33 µmol/L and 10.00 µmol/L MCL3 groups, respectively [Figure 4]. While MCL3 did not influence the cell proliferation at these concentrations for 24 h, according to results of the MTT assay. The data showed MCL3 inhibited the invasion of G422 cells \textit{in vitro}.

**MCL3 inhibited the tube formation of EA. hy926 cells**

The influence of MCL3 on the formation of vessels was explored by a capillary-like tube formation assay. The growth of new microvessels was inhibited in EA. hy926 cells after the treatment of various concentrations of MCL3. The inhibition rates were 24.8% \((P < 0.01)\), 81.7% \((P < 0.0001)\), and 91.9% \((P < 0.0001)\) in the 3.33 µmol/L, 10.00 µmol/L, and 30.00 µmol/L MCL3 groups, respectively [Figure 5]. These results indicated MCL3 had an inhibitory effect on \textit{in vitro} tube formation.

**MCL3 inhibited G422 tumor growth \textit{in vivo}**

The subcutaneously transplanted G422 xenograft model was used to determine the anti-tumor activity of MCL3 \textit{in vivo}. The results indicate that MCL3 suppressed the tumor growth at a dose-dependent manner in a subcutaneously transplanted G422 xenograft mice model [Figure 6], and the inhibition rate was 79.0% \((P < 0.001)\) in tumor weight at 40.0 mg/kg [Table 2].

**MCL3 suppressed the G422 tumor cell proliferation and angiogenesis \textit{in vivo}**

PCNA is the marker used to calculate cell proliferation rate. HIC staining showed that the lowest level of PCNA protein expression was detected in tumors treated with 40.0 mg/kg MCL3 compared to the control [Figure 7a], while the inhibition rate was 36.3% \((P < 0.001)\). The study used CD34 to evaluate the differences between MVD in the control and the 40.0 mg/kg MCL3 groups. Decreased tumor MVD was noted in the MCL3 group with an inhibition rate of 66.1% \((P < 0.5)\), which contrasted with the control [Figure 7b]. The results showed that MCL3 suppressed tumor cell proliferation and angiogenesis in G422 xenograft model.

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**Table 1: Summary of IC\textsubscript{50} values for MCL3 on different cancer cell lines**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC\textsubscript{50} (mol/L)</th>
<th>MCL3</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGC-803</td>
<td>(1.31\pm0.46)\times10^{-5}</td>
<td>(1.27\pm0.06)\times10^{-10}</td>
<td></td>
</tr>
<tr>
<td>BGC-823</td>
<td>(1.27\pm0.39)\times10^{-5}</td>
<td>(6.92\pm1.26)\times10^{-11}</td>
<td></td>
</tr>
<tr>
<td>HGC27</td>
<td>(8.72\pm0.83)\times10^{-6}</td>
<td>(7.30\pm5.46)\times10^{-10}</td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H1650</td>
<td>(1.18\pm0.34)\times10^{-6}</td>
<td>(4.34\pm0.78)\times10^{-7}</td>
<td></td>
</tr>
<tr>
<td>NCI-H460</td>
<td>(1.23\pm0.56)\times10^{-6}</td>
<td>(2.36\pm0.26)\times10^{-10}</td>
<td></td>
</tr>
<tr>
<td>Colon cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT15</td>
<td>(1.74\pm0.58)\times10^{-5}</td>
<td>(4.00\pm0.35)\times10^{-7}</td>
<td></td>
</tr>
<tr>
<td>HCT-8</td>
<td>(6.56\pm1.77)\times10^{-6}</td>
<td>(2.25\pm1.05)\times10^{-7}</td>
<td></td>
</tr>
<tr>
<td>Renal cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caki-1</td>
<td>(1.09\pm0.26)\times10^{-5}</td>
<td>(1.73\pm0.39)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>ACHIN</td>
<td>(1.30\pm0.03)\times10^{-5}</td>
<td>(1.95\pm0.41)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>(2.34\pm0.41)\times10^{-5}</td>
<td>(5.79\pm1.64)\times10^{-11}</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>(1.12\pm0.38)\times10^{-5}</td>
<td>(5.78\pm4.95)\times10^{-9}</td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capan2</td>
<td>(1.08\pm0.13)\times10^{-5}</td>
<td>(1.69\pm0.62)\times10^{-7}</td>
<td></td>
</tr>
<tr>
<td>BxPc3</td>
<td>(1.89\pm0.37)\times10^{-5}</td>
<td>(2.26\pm0.51)\times10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Mia-PaCa2</td>
<td>(6.68\pm0.79)\times10^{-6}</td>
<td>(5.55\pm1.23)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>Glioblastoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-87 MG</td>
<td>(6.44\pm1.80)\times10^{-6}</td>
<td>(1.71\pm0.31)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>U251</td>
<td>(1.48\pm0.93)\times10^{-5}</td>
<td>(2.84\pm1.42)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>Hs683</td>
<td>(1.89\pm0.41)\times10^{-5}</td>
<td>(1.66\pm0.37)\times10^{-5}</td>
<td></td>
</tr>
<tr>
<td>G422</td>
<td>(8.94\pm1.33)\times10^{-6}</td>
<td>(1.15\pm0.24)\times10^{-4}</td>
<td></td>
</tr>
</tbody>
</table>

Taxol was used as positive control for gastric cancer, lung cancer and breast cancer. Sorafenib was used as positive for renal cell carcinoma. TPT was used as positive control for colon cancer. Gemcitabine was used as positive control for pancreatic cancer. Iressa was used as positive control for glioblastoma. TPT: Topotecan

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**Figure 2**: IC\textsubscript{50} values of U87MG, U251, Hs683 and G422 cell lines treated with MCL3

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**Figure 3**: The effect of MCL3 on G422 cell apoptosis. Each graph represents the mean, and the error bars represent standard deviation. *\(P < 0.05\), **\(P < 0.001\) compared with control group.
MCL3 inhibited G422 glioblastoma growth mediated by NF-κB/IL-6/Stat3 signaling pathway

Du, et al.  MCL3 inhibited G422 glioblastoma growth mediated by NF-κB/IL-6/Stat3 signaling pathway

A dual-luciferase reporter assay was established to explore the effect of MCL3 on NF-κB and Stat3 transcription activity. The results showed that MCL3 inhibited NF-κB and Stat3 transcription activity [Figure 8]. The inhibition rate of NF-κB was 43.6% (P < 0.05) with 10.00 μmol/L MCL3, while the rate of Stat3 was 24.2% (P < 0.001) with 12.50 μmol/L MCL3, compared to the control group [Figure 8].

MCL3 decreased nuclear factor kappa B/Stat3 transcriptional activity

To elucidate the mechanism of MCL3, we analyzed the protein levels related to NF-κB/IL-6/Stat3 signaling pathway using western blotting. In vivo, 40.0 mg/kg MCL3 inhibited the phosphorylation of NF-κB, mediated by the suppression of phosphorylated IκBα, leading to decreased nuclear p-NF-κB [Figure 9a], and inhibited the expression of IL-6 regulated by NF-κB. Consequently, phosphorylation of Stat3 decreased [16] resulting in the downregulation of Stat3 and NF-κB target genes, including hypoxia inducible factor (HIF), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, MMP-9, Bcl-2, Bcl-xL and Mcl-1 [25-29] at 40.0 mg/kg MCL3 [Figure 9b and c].

This study evaluated the in vitro protein expression of G422 cells after treatment with MCL3 for 72 h using the western blot assay. The results demonstrated that 10.00 μmol/L MCL3 decreased the phosphorylation of NF-κB and IκBα in G422 cells, and decreased nuclear p-NF-κB was observed, resulting in the low expression of IL-6 [Figure 10a]. Finally, decreased phosphorylation of Stat3 was observed in G422 cells, and in nuclear p-NF-κB [Figure 10a and b]. Moreover, the target gene
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Discussion

Radiotherapy combined with TMZ is the first-line treatment for GBM, but it could cause resistance and recurrence due to the heterogeneity.[30,31] Furthermore, surgical resection was limited to the tumor location or the patient's poor physical status. Available therapeutic treatments and specific targets should be improved for GBM patients.[2]

TCM presents several advantages for cancer treatment, such as improving the anti-tumor effect of chemotherapy, reducing expression of Stat3 and NF-κB was reduced, including HIF, VEGF, MMP2, Bcl-2, and Mcl-1 [Figure 10c].

Table 2: The effect of MCL3 on the tumor growth in subcutaneously transplanted G422 xenograft model

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Animals (n) Begin/end</th>
<th>Body weight/g Begin</th>
<th>Body weight/g End</th>
<th>Tumor weight end/g</th>
<th>Inhibition/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6/6</td>
<td>16.9±0.6</td>
<td>31.7±3.2</td>
<td>3.24±0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMZ</td>
<td>30.0</td>
<td>6/6</td>
<td>17.3±0.7</td>
<td>33.2±1.9</td>
<td>0.94±0.47***</td>
<td>70.9</td>
</tr>
<tr>
<td>MCL3</td>
<td>10.0</td>
<td>6/6</td>
<td>16.9±0.4</td>
<td>33.2±1.8</td>
<td>2.17±0.70*</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>6/6</td>
<td>17.0±0.7</td>
<td>30.6±1.3</td>
<td>1.75±0.40***</td>
<td>45.9</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>6/6</td>
<td>16.9±0.8</td>
<td>31.6±2.1</td>
<td>0.68±0.22***</td>
<td>79.0</td>
</tr>
</tbody>
</table>

*P<0.05, ***P<0.001 compared with control group. TMZ: Temozolomide

Figure 7: Detection of proliferating cell nuclear antigen and CD34 expression in G422 xenograft tumor tissues analyzed by immunohistochemical assay. (a) MCL3 lowered the proliferation index evaluated by proliferating cell nuclear antigen staining (×20), ***P < 0.001 compared with control group. (b) Decreased microvessel density was observed in MCL3-treated group (×20), *P < 0.05 compared with control group

Figure 8: The effect of MCL3 on the nuclear factor kappa B/Stat3 signal pathway activity. (a) MCL3 inhibited nuclear factor kappa B transcriptional activity; (b) MCL3 inhibited Stat3 transcriptional activity. *P < 0.05, ***P < 0.001 compared with control group
the chemotherapeutic drug resistance, and enhancing immune function. TCM combined with chemotherapy could enhance drug efficacy and decrease toxicity, and could thus serve as an effective adjuvant cancer therapy.\cite{4}

Studies show that PTL inhibited the GBM growth via inhibiting NF-κB. ACT001, a derivative of PTL, modulated NF-κB to inhibit GBM growth, and ACT001 is currently in phase I clinical trials for advanced GBM treatment in China and Australia.\cite{32} MCL3 was synthesized with a three-step reaction from the natural product PTL. In the present study, the effects of MCL3 on the proliferation, invasion, and angiogenesis GBM were detected \textit{in vitro} and \textit{in vivo}. Results demonstrated that MCL3 inhibited GBM cell (G422, U-87 MG, U251 and Hs683 cell lines) proliferation \textit{in vitro}, and G422 GBM growth \textit{in vivo}. In addition, MCL3 suppressed the invasion of G422 cells, and suppressed tube formation in EA.hy926 cells.

Previous studies have shown that activated NF-κB increases the expression of IL-6, and IL-6 could lead to the activation of Stat3.\cite{13,33} \textit{In vitro} and \textit{in vivo} results have concurred that MCL3 inhibits the phosphorylation of nuclear mediated NF-κB via the suppression of phosphorylated IκB.\cite{15} and decreases the expression of IL-6 regulated by NF-κB.\cite{34} Consequently, the phosphorylation of IL-6-induced Stat3 was found to be reduced.\cite{35} MCL3 inhibited G422 tumor growth
through the inhibition of the NF-κB/IL-6/Stat3 signaling pathway.

NF-κB and Stat3 have been reported to be the significant transcription factors regulating the downstream genes associated with cell proliferation, apoptosis, invasion, and angiogenesis in cancer. A dual-luciferase reporter assay suggested that MCL3 inhibited the transcription activities of NF-κB and Stat3. Previous studies have suggested that NF-κB and Stat3 determine the expression of HIF-1α and VEGF, which play essential roles in vessel formation. Western blotting performed in the current study supported this evidence as MCL3 decreased the expression of p-NF-κB and p-Stat3, then reduced the levels of downstream proteins HIF-1α and VEGF in vivo and in vitro, which were associated with angiogenesis. Cancer cell invasion and metastasis could accelerate based on critical compounds for invasion, such as MMP2, MMP9. Other studies suggested that NF-κB activation regulated the expression of MMP9, while Stat3 enhanced the expression of MMP2, which is linked to metastatic activity. The study is in agreement with these findings, as the inhibition of p-NF-κB and p-Stat3 of MCL3 decreased the expression of its downstream proteins MMP9 and MMP2, in vitro and in vivo. Moreover, the inhibition of p-NF-κB and p-Stat3 decreased the levels of downstream proteins Bcl-2, Bcl-xl and Mcl-1, which were associated with cell apoptosis. The results showed that MCL3-induced apoptosis, suppressed tumor proliferation, invasion, and angiogenesis in G422 GBM, mediated by the NF-κB/IL-6/Stat3 signaling pathway. Furthermore, the IHC results also confirmed that tumor proliferation and angiogenesis were suppressed with the treatment of MCL3 in G422 xenograft tumor model.

CONCLUSION
The results demonstrated that MCL3 could induce apoptosis, inhibit G422 GBM growth, metastasis, and angiogenesis via the suppression of the NF-κB/IL-6/Stat3 signaling pathway. On the basis of this data, we suggest that MCL3 may be an effective treatment component for GBM.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES


42. Yu RX, Yu RT, Liu Z. Inhibition of two gastric cancer cell lines induced by fucoxanthin involves downregulation of Mel-1 and STAT3. Hum Cell 2018;31:50-63.