Anti-Glioma Effects of Ligustilide or n-Butylphthalide on Their Own and the Synergistic Effects with Temozolomide via PI3K/Akt Signaling Pathway

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Background: Ligustilide (LIG) and n-butylphthalide (NBP) have neuroprotective effects in cerebral ischemia; however, their roles in gliomas are not well-known. This study aimed to explore the anti-glioma effects of LIG and NBP individually and the synergistic effects of temozolomide (TMZ) via the PI3K/Akt Signaling Pathway.

Materials and Methods: Cytotoxicity of LIG and NBP alone and in combination with TMZ in U251 cells was determined using the CCk-8. The effect of compounds alone or in combination on cell migration was detected using the wound healing assay, and the invasion was evaluated by transwell assays, respectively. Cell apoptosis was quantified by flow cytometry and the changed expressions of proteins were detected by Western blotting.

Results: The results showed that LIG and NBP significantly inhibited the growth of U251 cells at concentrations of 4–10 µg/mL and 1.5–6 µg/mL in a dose-dependent manner (p<0.05, p<0.01). The combination of 20 µg/mL TMZ with LIG in the concentration range of 4–10 µg/mL or with NBP of 0.5–6 µg/ml achieved synergistic effect towards U251 cells. LIG and NBP, alone or in combination with TMZ, markedly inhibited cell invasion (p<0.001) and enhanced apoptosis (p<0.05). The combination of TMZ with LIG or NBP markedly inhibited cell migration (p<0.001). Western blot analysis showed that LIG, NBP, and TMZ, alone and in combination, significantly decreased the expression of Bel-2, p-PI3K, and p-Akt, and increased the expression of Bax.

Conclusion: Both LIG and NBP exert anti-glioma effects on their own through the PI3K/Akt pathway and enhance TMZ-mediated anti-glioma efficiency via the same pathway.

Keywords: ligustilide, n-butylphthalide, glioma, synergistic effect, PI3K/Akt signaling pathway

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor, with a median survival of less than one year.1 In the past 30 years, the incidence of glioma has been increasing at a rate of 1–2% per year.2 Current approaches to therapy include surgery, radiotherapy, and chemotherapy, as well as emerging gene therapy and immunotherapy in recent years; however, the results are unsatisfactory.3 Temozolomide (TMZ) is an oral alkylating agent and has been widely applied as an effective first-line chemotherapeutic agent for the treatment of glioma patients.4 However, TMZ resistance is one of the main reasons for glioma treatment failure. Drug combinations are one of the effective means to alleviate drug resistance and improve therapeutic effect of glioma,5 such as the combination of TMZ with an inhibitor of histone methyltransferase G9a BIX01294,6 and the combination of TMZ with a prototype cyclooxygenase (COX-2) inhibitor NS-398.7 The mechanism of the resistance to TMZ is various within the same tumor, and therefore it is a challenge to select which drug is compatible with TMZ.
Chuanxiong (*Ligusticum chuanxiong* Hort., Umbelliferae), a commonly used Chinese medicinal herb with hemodynamic and analgesic effects, has also been used in traditional Chinese medicine compound prescriptions to treat brain tumors, such as the Wenyang Tongqiao decoction, JiuweiTongqiao decoction, Naoliu power, XuefuZhuyu decoction. The essential oil of chuanxiong inhibited glioma angiogenesis, cell migration, and invasion via the EGFR/VEGF-A signaling pathway. Our previous research also showed that chuanxiong essential oil could enhance TMZ-induced anti-cancer efficiency in vivo. As the largest proportion of chuanxiong essential oil, phthalide contains a large amount of ligustilide (LIG) and n-butylphthalide (NBP); its chemical structure is shown in Figure 1. LIG significantly reduced T98G cells’ migration, inhibited the secretion of vascular endothelial growth factor A (VEGFA) from prostate

![Graphical Abstract](https://doi.org/10.2147/OTT.S432901)

**Figure 1** The chemical structures of ligustilide (LIG, a) and n-butylphthalide (NBP, b).
Interestingly, LIG showed little cytotoxicity to normal hepatocytes but restrained cell proliferation and migration, and promoted apoptosis of hepatocellular carcinoma cells. NBP in the form of a soft capsule and infusion drip was approved for marketing in 2002 to treat strokes. Recently, NBP was found to repress the proliferation of lung cancer cells and significantly reduce tumor volume and weight in vivo. However, it is unclear whether LIG and NBP have anti-tumor effects on glioma and whether they can play a synergistic role in combination with TMZ.

U251 cells are a type of human glioblastoma cell line that are commonly used in research studies related to brain tumors. These cells have several characteristics, including: (1) They are highly invasive and can migrate through tissue barriers. (2) They have a high rate of proliferation, meaning they can rapidly divide and replicate. (3) They have the ability to form tumors in vivo and in vitro. (4) They express several markers associated with glioblastoma, such as GFAP and vimentin. (5) They are resistant to chemotherapy and radiation therapy, which makes them a useful model for studying drug resistance mechanisms.

Overall, U251 cells are a valuable tool for studying the biology of glioblastoma and developing new therapies for this aggressive type of brain cancer. Therefore, in this paper, we adopted U251 cells to study the effects of LIG and NBP on the antitumor effects of TMZ.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays an important role in various cell physiological and pathological processes, including cell growth, survival, motility, DNA repair, and metabolism. Aberrant activation of PI3K-protein-serine-threonine kinase (Akt) pathway is closely associated with the occurrence, proliferation, apoptosis, invasion, metastasis, and multi-drug resistance in cancer cells, etc. Blocking the PI3K/Akt pathway may induce glioma cell apoptosis and inhibit tumor cell metastasis and proliferation. Therefore, the aims of this study were to (1) explore the anti-tumor effects of LIG and NBP on the glioma cell line U251 in vitro, and elucidate the mechanisms related to the PI3K/Akt signaling pathway; (2) investigate the synergistic anti-tumor effect of TMZ in combination with LIG or NBP and elucidate the mechanisms to provide a theoretical basis for clinicians to choose treatment measures.

Materials and Methods

Chemicals and Reagents

Human glioma U251 cells were obtained from ZhongqiaoXinzhou Biotechnology Co., Ltd. (Shanghai, China); D-HanksTrypsin-EDTA, Dimethylsulfoxide (DMSO), 0.1% crystal violet and PBS solution were obtained from Solarbio (Beijing, China); Annexin V conjugated to fluorescein-isothiocyanate (Annexin V-FITC), Cell Counting Kit 8 assay and tissues fixation solution were bought from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China); Dulbecco’s modified Eagle’s medium (DMEM) high-sugar complete medium was purchased from Shanghai ZhongqiaoXinzhou Biotechnology Co. Ltd. (Shanghai, China); LIG was purchased from Chengdu Ruifensi Biotechnology Co. Ltd. (Chengdu, China); NBP was purchased from Chengdu Lemeitian Pharmaceutical Technology Co., Ltd. (Chengdu, China); TMZ was purchased from Shanghai Yuanye Biotechnology Co., LTD (Shanghai, China); bicinchoninic acid (BCA) assay kit was purchased from Solarbio (Beijing, China); primary anti-bodies against Bax, Bcl-2, Akt, phosphorylatedAkt (Ser473, p-Akt), and GAPDH were purchased from Annoron Biotechnology Co., Ltd (Beijing, China); anti-PI3Kp8 5was purchased from Hangzhou HuaAn Biotechnology Co., Ltd (Hangzhou, China); anti-phosphoPI3K (Tyr607, p-PI3K) was purchased from Affinity Biosciences (Beijing, China); and secondary anti-bodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell Lines and Cell Culture

U251 cells were cultured in DMEM high-sugar complete medium with 10% fatal bovine, 1% streptomycin (100 μg/mL) and 1% penicillin (100 U/mL), and maintained in a humidified atmosphere of 5% CO₂ at 37 °C.

Cell Viability Analysis Detected by CCK-8 Assay

The effects of LIG, NBP, and TMZ on the growth of U251 cells were determined using a CCK-8 assay. LIG, NBP, TMZ, LIG-TMZ, and NBP-TMZ were dissolved and diluted in DMEM supplemented with 1% DMSO. U251 cells were seeded in 96 microtiter plates with a total culture medium of 100μL and a density of 8×10⁴ cells/mL, and then the plates were...
incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. After 24 h, the medium was removed and replaced with culture medium containing different compounds. After incubation for 24 hr, the medium was removed, and 100 µL fresh medium supplemented with 10% CCk-8 was added into each well and incubated for 20 min. Absorbance was measured at 490 nm using a Multiskan Go microplate reader (BioTek, Winoski, VT, USA). The absorption of wells containing compounds and cells was recorded as A<sub>sample</sub>, and the absorption of wells with the same concentration of compounds but without cells was recorded as A<sub>blank</sub>. The absorption of wells containing cells but no compounds was recorded as A<sub>control</sub>. The average absorbance of six measurements for each compound is represented as a percentage of the absorbance of the untreated control and plotted against the complex concentration.

**Cell Migration Determined by the Wound Healing Assay**

U251 cells were inoculated into 6-well plates at a density of 1×10<sup>5</sup> cells/mL and cultured until the cell density reached 80%–90%. Sterile of 200µL was used to scratch the plate surface vertically and achieve a similar cell wound width. Fresh medium supplemented with different compounds was added after removing the original culture medium. The scratch area in each group was recorded at 0 and 24 h using an inverted microscope, respectively. The wound-healing area was measured using a Nikon Eclipse TS10 inverted microscope (Nikon, Japan). Each experiment was performed in triplicates.

**Cell Invasion Detected by Transwell assays**

The substrate and pre-cooled serum-free solutions were uniformly coated on the surface of the upper chamber of transwell at a ratio of 1:9 for 6 h. Then, 200 µL of DMEM was added to hydrate the basement membrane for 2h in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. After removing the original culture medium, U251 cells treated with different compounds were seeded in the upper chamber with a total culture medium of 200µL and a density of 4×10<sup>5</sup> cells/mL, and 700 µL of culture medium without cells or compounds was added to the lower chamber. After incubation for 24 h, the U251 cells in the upper chamber were lightly removed and the chamber was fixed with fixative for 20 min. The fixative in chamber was removed, and the membrane was stained with 1% crystal violet for 15 min. The number of cells passing through the matrix glue was photographed and counted under a microscope. Crystal violet stained positive cells that pass through the matrix glue are considered to be invading cells.

**Cell apoptosis Determined by Annexin V-FITC/PI Double Staining Assay**

An annexin V-FITC/PI cell apoptosis detection kit was used to observe the effects of the compounds on the apoptosis of U251 cells. Cells were inoculated into 6-well plates at a density of 2×10<sup>5</sup> cells/mL and cultured until the cell density reached 80%–90%. Fresh medium supplemented with different compounds was added after removing the original culture medium. After incubation for 24 h, cells at a density of 2×10<sup>5</sup> cells/mL in 1×binding buffer (100 µL) were incubated with PI (5 µL) and Annexin V-FITC (5 µL) at room temperature for 15 min. Then, 1×binding buffer (400 µL) was added and the apoptosis level of glioma cells was investigated and analyzed using a BDFACSVerseflow cytometer (BD, USA).

**Protein Expressions Detected by Western Blot analysis**

Total protein was extracted from U251 glioma cells after 24 h treatment with different compounds, and its concentration was determined with BCA kit. Proteins were extracted from whole cell lysates, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. The following primary antibodies were used: rabbit anti-Bax (1:5000), anti-Bcl-2 (1:5000), anti-PI3K (1:500), anti-p-PI3K (1:500), anti-Akt (1:1000), and anti-p-Akt (1:1000). The membranes were then incubated with horseradish peroxidase-conjugated secondary anti-bodies (1:5000). The transferred proteins were incubated with an enhanced chemiluminescence substrate solution and visualized using ImageJ software (Bio-Rad). Relative expressions of proteins were quantified densitometrically using Quantity One software (Bio-Rad Laboratories, Richmond, CA, USA) and calculated according to the reference band of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
Statistics Analysis

The Viability of cells in the CCK-8 assay was calculated by using the following Equation 1:

\[
\text{Viability}(\%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100
\]  

Equation 1

Experimentally derived in vitro data were demonstrated as the mean ± standard deviation. Statistical analyses were performed using Prism 7.0 (GraphSoftware, San Diego, CA, USA) or SPSS 26.0 (IBM, Armonk, NY, USA). Data were analyzed by one-way analysis of variance, and the differences among multiple groups compared to the control or TMZ groups were analyzed by the least significant difference test. \(P<0.05\) was considered statistically significant.

Results

Effects of Compounds Alone or in Combination on U251 Cell Death

The effects of LIG and NBP alone or in combination with TMZ on the proliferation of U251 cells were evaluated using the CCK-8 assay (Figure 2). The TMZ group significantly inhibited growth of U251 cells in the concentration range of 20–200 \(\mu\)g/mL, and 20 \(\mu\)g/mL of TMZ was used for subsequent compatibility tests. Compared to the control group, both LIG and NBP significantly inhibited the growth of U251 cells in the concentration range of 4–10 \(\mu\)g/mL and 1.5–6 \(\mu\)g/mL in a dose-dependent manner \((p<0.05, p<0.01)\). In combination therapy, LIG or NBP significantly enhanced TMZ inhibition of cell growth when compared with the TMZ alone group \((p<0.001)\). The combination of 20 \(\mu\)g/mL TMZ with LIG in the concentration range of 4–10 \(\mu\)g/mL or with NBP of 0.5–6 \(\mu\)g/mL achieved enhanced effects against U251 cells.

The Q value method of Zhengjun Jin was used to analyze the synergistic interaction of the combination group, \(^{26-28}\) and it was shown in Table 1. The \(Q<0.85\) and \(Q\geq1.15\) indicate antagonism and synergy, respectively, whereas additivity is indicated by \(0.85\leq Q<1.15\). And the formula is \(Q = E_{(a+b)}/(E_a+E_b-E_a\times E_b)\), where \(E_{(a+b)}, E_a\) and \(E_b\) are average effect of

**Figure 2** Effects of TMZ, LIG and NBP alone and in combination on survival rate of U251 cells. Each value represents the mean ± SD of six independent experiments. Differs from control group, ***\(p<0.05\), ****\(p<0.01\), ####\(p<0.001\). Differs from TMZ alone group, ###\(p<0.001\).
combination treatment, effect of drug A only and effect of drug B only, respectively. The results showed that the Q values of the combination treatment of TMZ with LIG at 4 or 6 µg/mL were 1.53 and 1.19, respectively. The Q values of the combination treatment of TMZ with different concentrations of NBP were all greater than 1.15, suggesting a synergistic effect. Since both LIG and NBP could play a synergistic role with TMZ at 6 µg/mL, this concentration was used in subsequent tests.

### Effects of Compounds Alone or in Combination On Cell Migration and Invasion

The effects of LIG and NBP, alone or in combination with TMZ, on the migration of U251 cells were evaluated using wound healing experiments, and the results are shown in Figure 3. Figure 3 shows that TMZ at 20 µg/mL and LIG and NBP at 6 µg/mL did not significantly inhibit U251 cell migration, while compared with the control group ($p > 0.05$). The

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Figure 3 Effects of TMZ, LIG and NBP alone or in combination on migration of U251 cells. Values are mean ± standard deviation (n=3). Differs from control group, ***$p<0.001$. 

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combination of 20 µg/mL TMZ with 6 µg/mL LIG or NBP markedly inhibited cell migration when compared with the control group \((p<0.001)\), indicating synergistic effects on glioma cell migration.

The invasion of U251 cells treated with TMZ, LIG, or NBP alone or in combination for 24 h was determined by transwell assay, and the results are shown in Figure 4. Figure 4 demonstrated that TMZ, LIG, and NBP markedly inhibited the invasion of U251 cells compared with the control group \((p<0.001)\). The combination of TMZ with LIG or NBP also markedly inhibited cell invasion \((p<0.001)\), indicating synergistic effects on glioma cell invasion.

Effects of Compounds Alone or in Combination on U251 Cell Apoptosis

The effects of LIG/NBP and/or TMZ on U251 cell apoptosis were evaluated using flow cytometric analysis, and the results are shown in Figure 5. Figure 5 shows that TMZ at 20 µg/mL supplemented with LIG or NBP at 6 µg/mL significantly enhanced U251 apoptosis compared with the control group \((p>0.05)\). The combination of TMZ with NBP markedly enhanced cell apoptosis while compared with the control group or compound alone group \((p<0.001)\), indicating that NBP in combination with TMZ had synergistic effects on glioma cell apoptosis.

Contributions of PI3K/Akt Signaling athways

The effects of LIG/NBP and/or TMZ on protein expression were investigated by Western blot analysis, and the results are shown in Figure 6. Figure 6 reveals a band of 21 kDa corresponding to Bax, a band of 26 kDa corresponding to Bcl-2, a band of 56 kDa corresponding to Akt, 58 kDa, 80 kDa corresponding to p-PI3K, and 84 kDa corresponding to PI3K. Compared with the control group, the expression of Bcl-2 in U251 cells was markedly down-regulated by the compounds alone or in combination \((p<0.01)\), while the expression of Bax was markedly up-regulated \((p<0.01)\), which may explain...
Figure 5 Effects of TMZ, LIG and NBP alone or in combination on apoptosis of U251 cells. Values are mean ± standard deviation (n=3). Differs from control group, **p < 0.01, ***p < 0.001.

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Figure 6 Effects of LIG, NBP and TMZ alone or in combination on the PI3K/Akt signaling pathways in U251 cells. Values are mean ± standard deviation (n=3). Differs from control group, *p < 0.05, **p < 0.01, ***p < 0.001.
the increase in the apoptosis rate and the decrease in the U251 cell survival rate. Compared with the control group, LIG, NBP, and TMZ alone and in combination significantly decreased the expression of p-PI3K and p-Akt, indicating that the compounds alone and in combination may promote the apoptosis of U251 glioma cells by inhibiting the PI3K/Akt signaling pathway.

**Discussion**

Emerging evidence indicates that LIG has anti-tumor effects on Ehrlich solid carcinoma (ESC), hepatocellular carcinoma (HCC), prostate cancer, breast cancer and brain tumor, whereas NBP exhibits anti-cancer activity in lung cancer. There are few reports on the therapeutic effects of LIG and NBP, especially NBP, on gliomas. Notably, LIG promoted apoptosis in ESC by significantly increasing the expression of Bcl2. It also dramatically inhibits the viability and migration of HCC and oral cancer cells. In this paper, we investigated the functions of LIG and NBP in U251 the proliferation, migration, invasion, and apoptosis. These results indicate that LIG and NBP significantly enhanced U251 glioma cell apoptosis and inhibited U251 cell viability and invasion. Interestingly, both LIG and NBP can inhibit neuronal apoptosis and improve the proliferation induced by cerebral ischemia.

One of the main steps in tumorigenesis and development is to escape apoptosis. And the occurrence and development of tumor is evasion of apoptosis. Therefore, the possible mechanism of apoptosis pathway as LIG and NBP in the treatment of glioma was investigated in this study. This process is regulated by a balance between the pro-apoptotic protein Bax and anti-apoptotic proteins Bcl-2 within cells. Our findings revealed LIG and NBP up-regulated Bax and down-regulated Bcl-2, respectively, leading to imbalance and affecting apoptosis. Bcl-2 is a well-known anti-apoptotic compound, and its deletion or down-regulation has been found in many tumor types. In addition, Bax promotes apoptosis by activating cytochrome C and caspase-3.

The PI3K/AKT signaling pathway plays an important role in regulating cell proliferation and apoptosis, which is often abnormally activated in cancer. PI3K is a member of the lipid kinase family, and its activation can catalyze the 3-hydroxyl phosphorylation of PIP2 to generate PIP3, which acts as the second messenger and recruits PDK1 and Akt to the plasma membrane, resulting in Akt activation. Activated Akt then promotes apoptosis by up-regulating Bcl-2 family proteins. In this study, we investigated the effects of LIG and NBP on PI3K/Akt pathway. Both LIG and NBP significantly decreased the expression of p-PI3K and p-Akt, indicating that they promote apoptosis of U251 glioma cells by inhibiting the PI3K/Akt signaling pathway. However, the effects of LIG and NBP on the expression of apoptotic executioner proteins, such as cleaved caspase-9 and cleaved caspase-3, were not detected in this study; therefore, further investigation is required.

TMZ is an oral-alkylating drug, whose molecular action is based on the formation of O6-methylguanine, and as a result, O6-methylguanine mispairs with thymine, resulting in cell cycle arrest at G2/M and consequent programmed cell death. However, drug resistance limits their antitumor efficacy. The combination of LIG and tamoxifen induces apoptosis in breast cancer cells and S and G2/M phase cell cycle arrest. Importantly, LIG restored the sensitivity of breast cancer cells to tamoxifen by reversing the MTA1/IFI16/HDACs complex mediated epigenetic repression of ERα, inhibiting autophagy, and accumulating DNA damages. Interestingly, LIG and NBP not only have anti-tumor effects but also synergistically inhibit the viability, migration, and invasion of glioma cells when combined with TMZ. Additionally, Western blot analysis indicated that the combined treatment with NBP and TMZ synergistically induced U251 cell apoptosis by inhibiting the PI3K/Akt signaling pathway and disturbing the imbalance of Bcl-2 family expression. These results suggest that both LIG and NBP may be chemo sensitizers that enhance TMZ-induced anti-cancer efficacy against human gliomas.

LIG and NBP have shown synergistic anti-tumor effects with TMZ in vitro cell models; however, further studies are needed to confirm the synergistic effects observed in animal models. In addition, the mechanism of the resistance to TMZ is various within the same tumor, such as DNA damage repair, DNA mismatch repair system, and base excision repair system, etc. In this paper, we confirmed that LIG and NBP have anti-glioma effects in coordination with TMZ through the PI3k-Akt signaling pathway, and whether they can also play synergistic roles through other pathways needs further study.
Conclusion
In summary, the results of the present study demonstrated that both LIG and NBP inhibited proliferation and invasion and induced apoptosis in U251 glioma cells by regulating the PI3K/Akt signaling pathway and that both LIG and NBP have the potential to enhance TMZ-mediated anti-cancer efficiency against human glioma through the PI3K/Akt signaling pathway, which validated that the combination of TMZ with LIG or NBP may be an effective way to treat human glioma. However, this requires further in vivo study.

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Disclosure
The authors declare that there is no conflict of interest in this work.

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