

# Inhibitory effects of novel isothiocyanate derivative on proliferation and invasion of glioma U251 cells and regulation of circRNAs and lncRNAs

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## ABSTRACT

**Gliomas proliferation and invasion plays the critical roles in the tumor progression. Isothiocyanate is the hydrolysis products of Brassica vegetables and suppress many types of tumor including gliomas. We design and synthesized an isothiocyanate derivative by computer-aided drug design base on alkylglycerone phosphate synthase (AGPS) to improve targeting and explore its effect on the proliferation, invasion, apoptosis, cell cycle, and the expression of circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) in U251 cell line. The isothiocyanate derivative suppressed proliferation and invasion by inducing apoptosis with improving the activity of caspase-3/8 and blocked the cell cycle at G2/M phase and regulating the expression of circRNAs and lncRNAs involved in the above biological behavior in human glioma U251 cells. Our results showed the effect of isothiocyanate derivative on the anti-tumor activity, which shows potential for application as a anti-glioma drug.**

## INTRODUCTION

Glioma is one of the most common malignant tumors seriously damaging human's health. Although major advances have been made in the study of relevant clinical treatments, the prognosis is still not ideal [1]. Tumor metastasis is one of the primary reasons for treatment failure. Clinically, nearly half of the patients were diagnosed with advanced glioma, and there was the metastasis [2]. Therefore, it is of great significance to develop new drugs to inhibit proliferation and metastasis. It was considered that circular RNAs (circRNAs) [3] and long non-coding RNAs (lncRNAs) [4] were an important role in tumor process, changed expression of them could improve or suppress the potential of proliferation and invasion in glioma. Therefore, it is considered that circRNAs and lncRNAs were important target of tumor, including glioma.

Isothiocyanate is the hydrolysis products of Brassica vegetables [5]. Many previous studies of isothiocyanate

were conducted on the apoptosis and inhibit proliferation and metastasis of tumor cells [6]. We also found that it could inhibit the expression of alkylglycerone phosphate synthase (AGPS) which was an oncogene and the proliferation and invasion of tumor cells can be inhibited and the drug sensibility of drug resistant cells can be improved by AGPS silencing in our previous study [7–8], meanwhile, we found that AGPS silencing could regulate the expression of circRNAs and lncRNAs of U251 cell line (data was not published).

We design and synthesized isothiocyanate derivative by CADD (computer-aided drug design) (data was not showed) targeting AGPS which was the new compound un-reported, predicted the ADME (absorption, distribution, metabolism, excretion) and toxicity, and investigates the inhibitory effect of the isothiocyanate derivative on the proliferation and invasion of glioma U251 cells, providing the basis for further development and clinical application of isothiocyanate derivative.

## RESULTS

### Virtual screening, ADME and toxicity prediction results

With Virtual screening and prediction, the isothiocyanate derivative can have a good combination with AGPS cavity and ADME/ toxicity, and amino acid residues form hydrogen bond and hydrophobic effect with nitrogen, sulfur atom and carbon sulfur covalent bond, meanwhile, the other substituent residues in micromolecules can form a certain hydrogen bonding interaction with their surrounding residues to a certain extent (Table 1). Ideal drug parameter reference range for ADME: a, 7.0~200.0; b, -2.0~6.5; c, -6.5~0.5; d, ~25 is bad, ~500 was good.

### Effects of isothiocyanate derivative on the proliferation and expression of AGPS in U251 cells

The proliferation inhibition rate of U251 cells with different concentrations of isothiocyanate derivative was shown in (Figure 1A). With the increase of drug concentration, the inhibitory effect gradually increased, with significant concentration - effect relation and the IC<sub>50</sub> was 73.4  $\mu$ M for 24 h. We used the non-toxic concentration (5  $\mu$ M) (inhibition rate <5%) and low-toxic concentration (10  $\mu$ M) (inhibition rate <15%) to following research to avoid the affect of cell proliferation inhibition, the 0  $\mu$ M was the control group. The mRNA of AGPS was not significant decreased with 5  $\mu$ M and 10  $\mu$ M isothiocyanate derivative treatment (Figure 1B).

### Effects of isothiocyanate derivative on the invasion of U251 cells

With the increase of the concentration of isothiocyanate derivative, the invasive cells were decreased. Compared with the control group, the results showed that the isothiocyanate derivative could inhibit the invasion of U251 cells with certain dependence of concentration (Figure 2).

### Effects of isothiocyanate derivative on the cell cycle and apoptosis of U251 cells

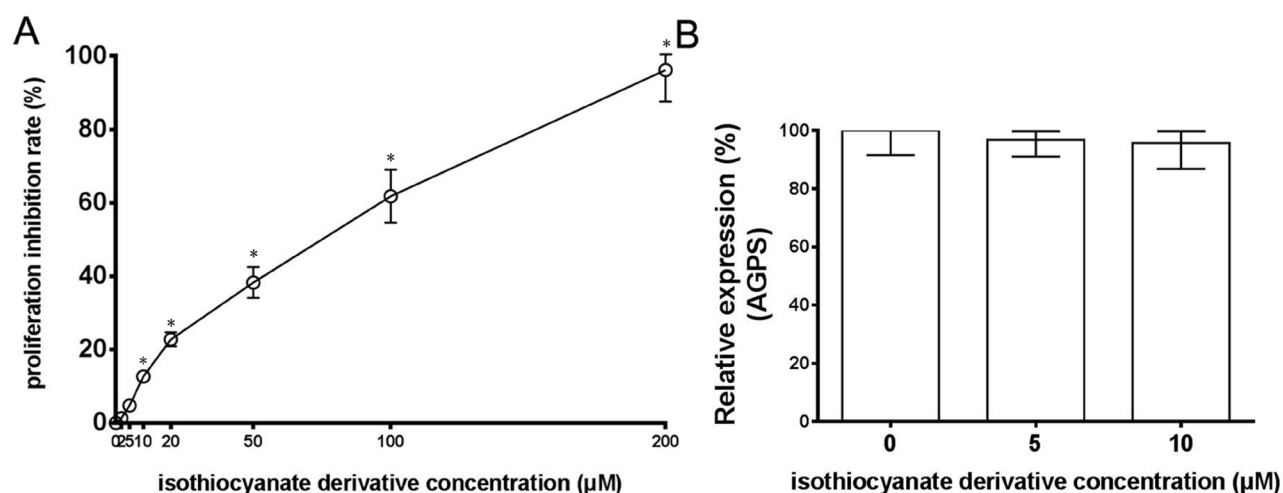
Compared with the control group, with the increase of drug concentration, the G2/M phase of U251 cells was increased (Figure 3A), and the apoptosis cell was significantly increased (Figure 3B), conforming to flow cytometry results. At same time, the activity of caspase-3/8 was improved (Figure 3C).

### Effects of isothiocyanate derivative on the expression of circRNAs and lncRNAs

After treatment of U251 cells with different concentrations of isothiocyanate derivative for 24 h, compared with the control group, with the increase of drug dosage, the expression of circRNAs and lncRNAs was significant differences, however, the expression of AGPS was not significant changed (Figure 4).

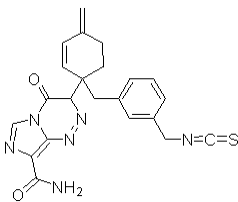
## DISCUSSION

One of the main reasons for the failure of glioma therapy is metastasis, and the control of metastasis is one of the key factors to determine the prognosis of



**Figure 1: Effects of isothiocyanate derivative on the proliferation and expression of AGPS in U251 cells.** (A) The effect of isothiocyanate derivative on the proliferation in U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ . (B) Effects of isothiocyanate derivative on the mRNA expression of AGPS in U251 cells.

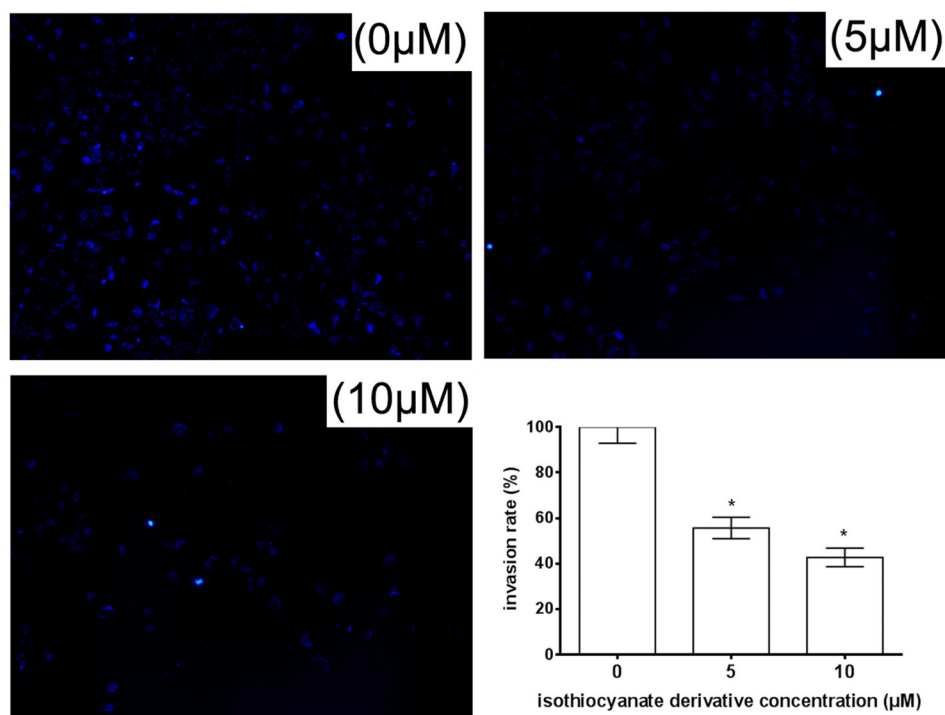
**Table 1: The structure and combination model of isothiocyanate derivative**

The structure and combination model							
Structure		2D combination model		3D combination model		scoring	
							

patients. The occurrence of tumor metastasis includes cell proliferation, adhesion, invasion and others, with complex mechanism [9]. Currently, the anticancer study of nature compound has been concerned [10]. However, there were also some weakness need to overcome. The structure reform was a method to improve compound property. Since a lower stability of isothiocyanate, we design its

derivative to improve the stability by CADD, meanwhile, we investigated the effect of isothiocyanate derivative on the proliferation and invasion in U251 cells.

With *vitro* experiment MTS assay, this experiment confirmed that isothiocyanate derivative can inhibit the proliferation of glioma U251 cell, with concentration dependence. Invasion was the key part of tumor

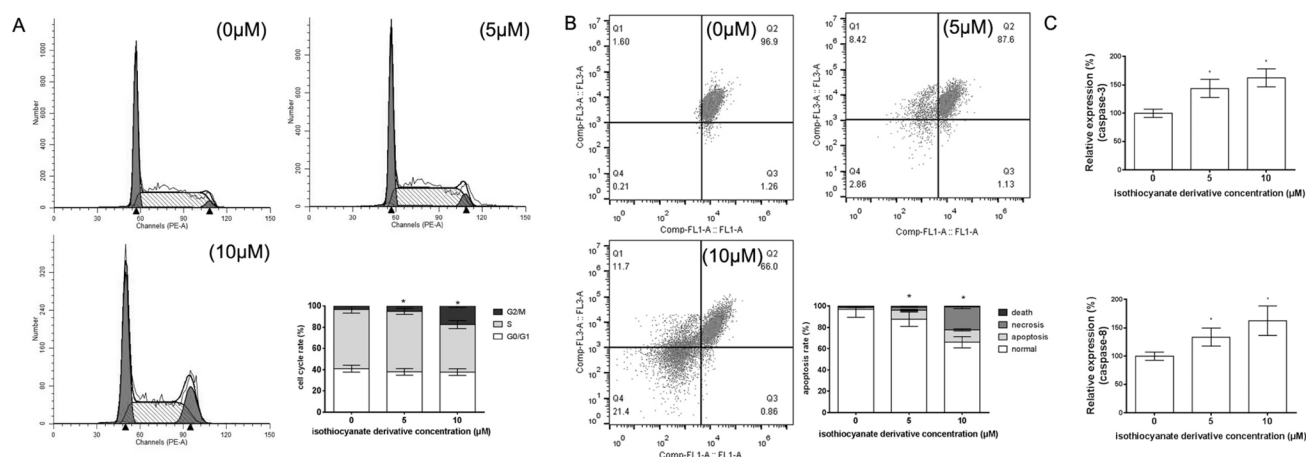


**Figure 2: Effects of isothiocyanate derivative on the invasion of U251 cells.** \*compared with 0 μM group,  $P < 0.05$ .

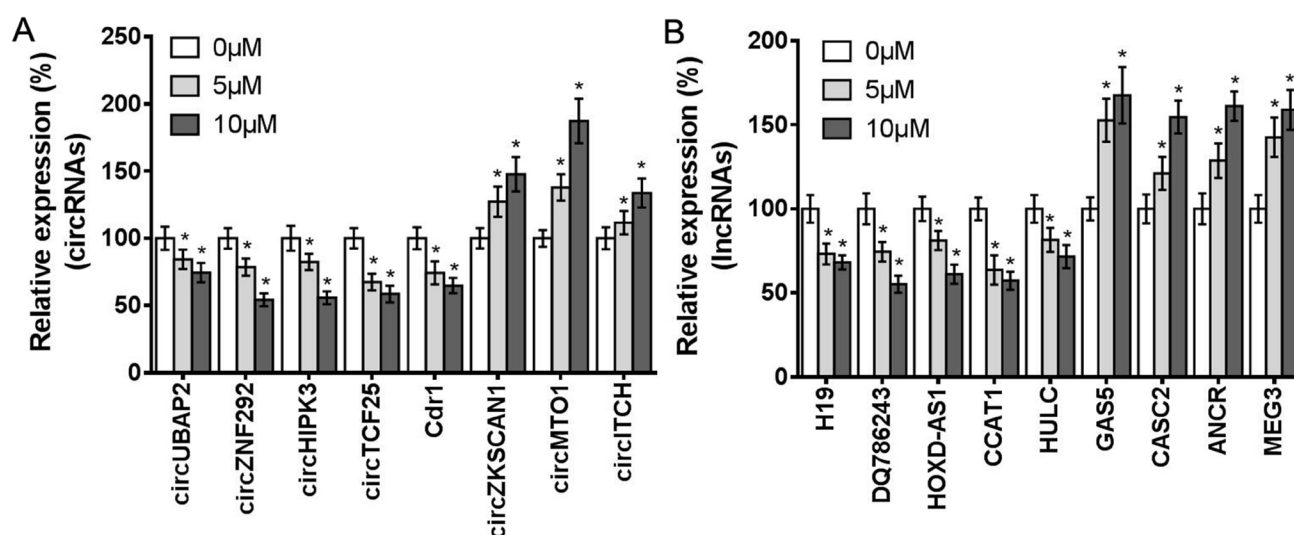
metastasis, we also found that isothiocyanate derivative can inhibit the invasion of glioma in non-toxic (5  $\mu$ M) and low-toxic concentration (10  $\mu$ M). However, the mRNA expression of AGPS was not significantly decreased, suggesting the anti-tumor of isothiocyanate derivative was not from the decreased expression of AGPS. Therefore, the results showed that isothiocyanate derivative was a potential anti-tumor drug which need to explore the mechanism. Cell cycle arrested and apoptosis should be the one of the mechanism of isothiocyanate derivative due to it could induce cell cycle arrested and apoptosis.

Tumor cells existed a variety of circRNAs and lncRNAs, playing a key role in promoting tumor cell

proliferation and metastasis [11–12]. Our study showed that AGPS silencing could regulate the expression of following circRNAs and lncRNAs of U251 cell line (data was not published) and we have confirmed that isothiocyanate derivative could suppress the expression of circRNAs circUBAP2, cZNF292, circHIPK3, circTCF25 and Cdr1, and lncRNAs H19, DQ786243, HOXD-AS1, CCAT1 and HULC, which were the oncogene [13–22]. Meanwhile, it improved the expression of circRNAs circZKSCAN1, circMTO1 and circITCH, and lncRNAs GAS5, CASC2, ANCR and MEG3, which were the tumor suppressor gene [23–30]. Above regulation of circRNAs and lncRNAs was same as the AGPS silencing.



**Figure 3: Effects of isothiocyanate derivative on the cell cycle and apoptosis of U251 cells.** (A) Effects of isothiocyanate derivative on the cell cycle of U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ . (B) Effects of isothiocyanate derivative on the apoptosis of U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ . (C) Effects of isothiocyanate derivative on the activity of caspase-3/8 of U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ .



**Figure 4: Effects of isothiocyanate derivative on the expression of circRNAs and lncRNAs of U251 cells.** (A) Effects of isothiocyanate derivative on the expression of circRNAs of U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ . (B) Effects of isothiocyanate derivative on the expression of lncRNAs of U251 cells. \*compared with 0  $\mu$ M group,  $P < 0.05$ .

In a word, isothiocyanate derivative inhibits the proliferation and invasion, induce cell cycle arrested and apoptosis, regulating the expression of circRNAs and lncRNAs may be one of its regulation mechanisms. We also explored the effect of the isothiocyanate derivative on the anti-tumor activity in hepatic carcinoma, lung cancer, breast cancer and so on, it will be showed in further article.

## MATERIALS AND METHODS

### Virtual screening

AGPS three-dimensional structure model (PDBID:2UUV) was downloaded from PDB Bank and the micromolecular structure was draw by ChemBioOffice2010. "CDOCKER ENERGY" was measured by Discovery Studio 3.5 and the ADME including logPo/w (oil-water partition coefficient), PSA (polarization surface area), logS (water solubility) and PMDCK (permeability Maden Darby Canine Kidney), meanwhile, toxicity including rodent carcinogenicity, mutagenicity, skin irritancy, ocular irritation, aerobic biodegradability, were predicted by Schrodinger Suite 2009 software.

### Cell culture

U251 cell line was provided by American Type Culture Collection (USA). The medium was the DMEM medium with 10% fetal bovine serum (FBS) (Gibco). The cells were cultured in the cell incubator with 5% CO<sub>2</sub> at 37° C.

### MTS assay

5 × 10<sup>3</sup> U251 cells suspension was added in 96-well plates for 24 h, and isothiocyanate derivative with different concentrations (0, 2, 5, 10, 20, 50, 100, 200 μM) was added for 24 h. Then the cell proliferation was measured by MTS assay. Inhibition rate/% = (1 - OD value<sub>treatment group</sub> / OD value<sub>control group</sub>) × 100%.

### Transwell assay

Matrigel was added on Transwell upper chamber. U251 cells (2 × 10<sup>4</sup> cells /well) with 5 and 10 μM isothiocyanate was added in the upper chamber, and the culture medium with 10% fetal bovine serum was added in the lower chamber for 16 h in 5% CO<sub>2</sub> at 37° C. Then the membrane was fixed with paraformaldehyde, stained with 100 ng/ml DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) for 10 min, the cells were counted under the microscope (× 200) at 488 nm with the average.

### Cell cycle assay

3 × 10<sup>5</sup> U251 cells suspension was added in 6-well plates for 24 h, and 5 and 10 μM isothiocyanate derivative was added for 24 h in 5% CO<sub>2</sub> at 37° C. Then cells were digested, fixed by 70% alcohol at 4° C, and cells were stained with propidium iodide (PI) at room temperature darkly. The cell cycle was measured by FACS Aria flow cytometry (BD Biosciences) at 488 nm.

### Cell apoptosis assay

3 × 10<sup>5</sup> U251 cells suspension was added in 6-well plates for 24 h, and 5 and 10 μM isothiocyanate derivative was added for 24 h in 5% CO<sub>2</sub> at 37° C. Then cells was digested and stained with Annexin V-FITC/PI at room temperature darkly. The cell apoptosis was measured by FACS Aria flow cytometry (BD Biosciences) at 488 nm.

### Caspase-3/8 activity assay

To investigate the cellular activity of caspase-3/8, 3 × 10<sup>5</sup> U251 cells suspension was added in 6-well plates for 24 h, and 5 and 10 μM isothiocyanate derivative was added for 24 h in 5% CO<sub>2</sub> at 37° C. The cellular activity of caspase-3/8 was measured according to the manual's instruction by FLICA Caspase Detection Kits (ImmunoChemistry Technologies) using a microplate reader.

### Realtime PCR assay

3 × 10<sup>5</sup> U251 cells suspension was added in 6-well plates for 24 h, and 5 and 10 μM isothiocyanate derivative was added for 24 h in 5% CO<sub>2</sub> at 37° C. Cell total RNA was extracted by TRIzol reagent (Invitrogen). Total RNA was then reverse transcribed and expression of mRNAs, circRNAs and lncRNAs were detected using real-time PCR assay. The PCR conditions were as follows: denaturation at 95° C for 10 min, followed by 40 cycles at 95° C for 15 s, 60° C for 60 s and a final elongation at 95° C for 15 s, 60° C for 60 s and 95° C for 15 s. The expression levels of the genes were normalized to that of the housekeeping gene β-actin, as a control. The full details of the primers used in these experiments are shown in (Table 2).

### Statistical analysis

The experimental data are statistically analyzed with SPSS 11.0 statistical software (SPSS Inc., IL, USA) and data is expressed with mean ± SD. The statistical analysis was performed using one-way ANOVA with the Tukey-Kramer multiple comparisons test. *P* < 0.05 was considered statistically significant.



**Table 2: The primers used in the study**

Gene	Primer sequences (5'–3')
AGPS	Forward: ACCAGATTCCTGGAGTTCA Reverse: GAACCACCAGGTCCTCGATA
DQ786243	Forward: AATCGGCTCTGGAAGGTGAA Reverse: CTGCTGTTCCGATGGTGTCTT
HOXD-AS1	Forward: GGCTCTTCCCTAATGTGTGG Reverse: CAGGTCCAGCATGAAACAGA
CCAT1	Forward: CATTGGGAAAGGTGCCGAGA Reverse: ACGCTTAGCCATACAGAGCC
HULC	Forward: CAGGAAGAGTCGTCACGAGAACCAG Reverse: CTTCTTGCTTGATGCTTTGGTCTGT
GAS5	Forward: CTTCTGGGCTCAAGTGATCCT Reverse: TTGTGCCATGAGACTCCATCAG
CASC2	Forward: GCACATTGGACGGTGTTC Reverse: CCC AGTCCTTCACAGGTCAC
ANCR	Forward: GACATTCCTGAGTCGTCTTCGAACGGAC Reverse: TAGTGCGATTTAGAGCTGTACAAGTTTC
MEG3	Forward: TTTTGTGCCCAAGGCTCCTGGA Reverse: AGGGACTCAAGGAGCCAGGTTA
circUBAP2	Forward: AGCCTCAGAAGCCAACCTCTTTG Reverse: TCAGGTTGAGATTTGAAGTCAAGAT
circZNF292	Forward: GCTCAAGAGACTGGGGTGTG Reverse: AGTGTGTGTTCTGGGGCAAG
circTCF25	Forward: CGGAATTCTGAAATATGCTATCTTACAGAGAGAGCGCTGTACAGCATGGA Reverse: CGGGATCCTCAAGAAAAATATATTCACCTCCAGGGAACATGGTGAGCGC
circHIPK3	Forward: TATGTTGGTGGATCCTGTTCGGCA Reverse: TGGTGGGTAGACCAAGACTTGTGA
circCdr1	Forward: GTGTCTCCAGTGTATCGGCG Reverse: TACTGGCACCACCTGGAAACC
circZKSCAN1	Forward: AGTCCCACTTCAAACATTCTGTCT Reverse: CACCTTCACTATTACGATACCATCC
circITCH	Forward: GCAGAGGCCAACACTGGAA Reverse: TCCTTGAAGCTGACTACGCTGAG
MTO1	Forward: GAGCTGTAGAAGATCTTATTC Reverse: CACAGGCCATCCAAGGCATC
β-actin	Forward: AGGCACCAGGGCGTGAT Reverse: GCCCACATAGGAATCCTTCTGAC

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None.

## CONFLICTS OF INTEREST

No potential conflicts of interest are disclosed.

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