

Carboxamide derivatives induce apoptosis in the U251 glioma cell line

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Abstract. Glioma is a malignant tumor that is frequently treated using chemotherapy. The aim of the present study was to examine the antitumor activity of two novel carboxamide derivatives in glioma, and investigate the underlying mechanisms. Two previously designed and synthesized carboxamide derivatives were selected and their effects on glioma cells were evaluated. Specifically, assays to evaluate proliferation, apoptosis, oxidation, caspase-3, -8 and -9 activity, and the expression of Bcl-2 and surviving in glioma cells were conducted. The carboxamide derivatives were revealed to inhibit proliferation, as well as to induce apoptosis and oxidative damage in glioma U251 cells. In addition, the carboxamide derivatives increased the activity of caspase-3, -8 and -9, and suppressed the expression of Bcl-2 and survivin. These findings demonstrate that the carboxamide derivatives displayed antitumor activity against glioma *in vitro*, which may have been mediated via the induction of oxidative damage and apoptosis.

Introduction

Glioma is a malignant tumor that is a threat to human health, with a median survival time of only 14.6-17 months for World Health Organization grade IV gliomas and an incidence rate of ~5/100,000 in China in 2011 (1,2). Chemotherapy remains the major therapeutic method for glioma treatment (3,4). Currently, temozolomide (TMZ) is used clinically for the treatment of glioma; however, its clinical application is limited due to toxicity (5). Therefore, a number of studies have been

initiated with the aim of developing a new antitumor drug with reduced toxicity (6,7).

A number of lipid synthesis-associated genes are involved in tumorigenesis (8). Previous studies have indicated that alkyl-glycerone phosphate synthase (AGPS) is a critical enzyme in ether lipids synthesis and it is upregulated in several types of cancer cells and primary tumors. It increases the malignancy of many types of tumor, including prostate cancer, melanoma, breast cancer and glioma by altering the balance of structural and signaling lipids which affect cancer pathogenicity (9). Therefore, the present research team hypothesized that AGPS may be a viable target for anticancer drugs, and designed and synthesized a novel series of AGPS-targeting carboxamide derivatives by computer-aided drug design (10,11). These derivatives include N2 and S2 (Fig. 1A and B).

There is an excellent docking score of N2 and S2 in our previous study (10). To examine the potential of these derivatives as novel anti-glioma drugs, their effects on glioma cells were examined in this study, in comparison with TMZ, and the mechanism underlying their effects was investigated.

Materials and methods

Compounds, cell lines and culture. The compounds N2 and S2 were synthesized by Werian Biotech Co. (Jinan, China). Human glioma U251 and H4 cell lines and the PC12 rat pheochromocytoma cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). The U251 and H4 cell lines were cultured in DMEM with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C and 5% CO₂. The PC12 cell line was cultured in Ham's F12K (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C and 5% CO₂.

MTS assays. U251, H4 and PC12 cells were cultured on 96-well plates (3x10³/well) overnight. Different concentrations (0, 10, 20, 50, 100 and 200 μM) of N2, S2 and TMZ (Sigma-Aldrich; Merck KGaA) were added to the cells, which were then cultured for 72 h at 37°C. A total of 20 μl MTS was added and cells were cultured for 4 h at 37°C. The optical density (OD) was then measured at a wavelength of 490 nm. Inhibition

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percentage was calculated as $(1 - \text{OD value}_{\text{treatment group}} / \text{OD value}_{0 \mu\text{M}}) \times 100$. 20 μM of the antioxidant N-acetyl-L-cysteine (NAC) (Beyotime Institute of Biotechnology) was added with N2, S2 and TMZ.

Flow cytometry assay. U251 cells (2×10^5 /well) were cultured in 6-well plates. The cells were cultured with N2 (50 and 80 μM), S2 (20 and 30 μM) or TMZ (10 and 20 μM) for 72 h at 37°C. Cells were then collected, and apoptosis was assessed using the Annexin V-FITC/PI kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol, with incubation for 15 min at room temperature in the dark. Apoptosis was measured by flow cytometry (FACS Aria; BD Biosciences) at a wavelength of 488 nm by Diva software (version 8.0.1; FACS Aria; BD Biosciences).

Reactive oxygen species (ROS) assay. ROS are responsible for oxidative damage (12). U251 cells (2×10^5 /well) were cultured in 6-well plates. Following the addition of N2 (25 and 40 μM), S2 (10 and 15 μM) or TMZ (5 and 10 μM), the cells were cultured for 72 h at 37°C. The cells were then collected and stained with 2,7-dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology). ROS were quantified using a microplate reader at a wavelength of 488 nm.

Caspase-3, -8 and -9 activity assays. U251 cells (2×10^5 /well) were cultured in 6-well plates with N2 (25 and 40 μM), S2 (10 and 15 μM) or TMZ (5 and 10 μM) for 72 h at 37°C. The caspase-3, -8 and -9 activities of the cells were then measured using caspase detection kits (cat. nos. 93, 99 and 912; Immunochemistry Technologies, LLC), with a microplate reader at a wavelength of 488 nm.

Western blot analysis. U251 cells (2×10^5 /well) were cultured in a 6-well plate with N2 (25 and 40 μM), S2 (10 and 15 μM) or TMZ (5 and 10 μM) for 72 h at 37°C. The cells were then lysed using cell lysis buffer (Beyotime Institute of Biotechnology), containing 20 mM Tris (pH 7.5), 150 mM NaCl and 1% Triton X-100, and total proteins were extracted. A total of 50 μg protein/lane determined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology) was separated via 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was then blocked using 1% bovine serum albumin (Beyotime Institute of Biotechnology) for 1 h at 37°C. The membrane was incubated with Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. no. sc-7382; 1:1,500) and survivin (Santa Cruz Biotechnology, Inc.; cat. no. sc-101433; 1:1,500) antibodies overnight at 4°C, and then incubated for 1 h at 37°C with mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc.; cat. no. sc-2357; 1:2,000). The membrane was visualized using Immobilon Western chemiluminescent horseradish peroxidase substrate (EMD Millipore, Billerica, MA, USA). β -actin (Santa Cruz Biotechnology, Inc.; cat. no. sc-81178; 1:5,000) was used as the loading control. Quantification was performed using Image J software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative PCR (RT-qPCR) assay. U251 cells (2×10^5 /well) were cultured in 6-well plates with N2 (25 and 40 μM), S2 (10 and 15 μM) or TMZ (5 and 10 μM)

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene	Primers
Bcl-2	Forward: 5'-CGTACAGTTCCACAAAGGCA-3' Reverse: 5'-ATGTGTGTGGAGAGCGTCAA-3'
Survivin	Forward: 5'-TCCGCAGTTTCCTCAAATTC-3' Reverse: 5'-GTTGCGCTTTCCTTCTGTC-3'
β -actin	Forward: 5'-AGGCACCAGGGCGTGAT-3' Reverse: 5'-GCCCACATAGGAATCCTTCTGAC-3'

Table II. IC₅₀ values of carboxamide derivatives and TMZ in PC12, H4 and U251 cell lines.

Compound	IC ₅₀ (μM)		
	PC12	H4	U251
N2	87.6	108.3	122.7
S2	42.3	53.8	47.4
TMZ	34.3	42.8	37.5

IC₅₀, half maximal inhibitory concentration; TMZ, temozolomide.

for 72 h at 37°C. Total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA was reverse transcribed using BeyoFast™ SYBR Green qPCR Mix kit including reverse transcriptase, buffer and dNTPs (Beyotime Institute of Biotechnology) and expression of the target genes were detected using a qPCR assay (cat. no. ABI7500; Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following conditions: Denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 60 sec and a final extension step at 95°C for 15 sec. Expression was normalized to β -actin. The full details of the primers used in these experiments are shown in Table I. The quantified results were calculated using the $2^{-\Delta\Delta C_t}$ method (13).

Statistical analysis. Experimental data is presented as the mean \pm standard deviation of three experimental repeats. SPSS statistical software (version 11.0; IBM Corp., Armonk, NY, USA) was used to perform a one-way analysis of variance with the least significant difference post hoc test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of carboxamide derivatives on the proliferation of U251 cells. The MTS assay results revealed that the carboxamide derivatives and TMZ inhibited the proliferation of U251, H4 and PC12 cells. However, the antiproliferative effect of the carboxamide derivatives was decreased compared with that of TMZ, as revealed by their higher half maximal inhibitory concentrations (Table II). This indicates that the carboxamide derivatives had a lower toxicity compared with TMZ. The

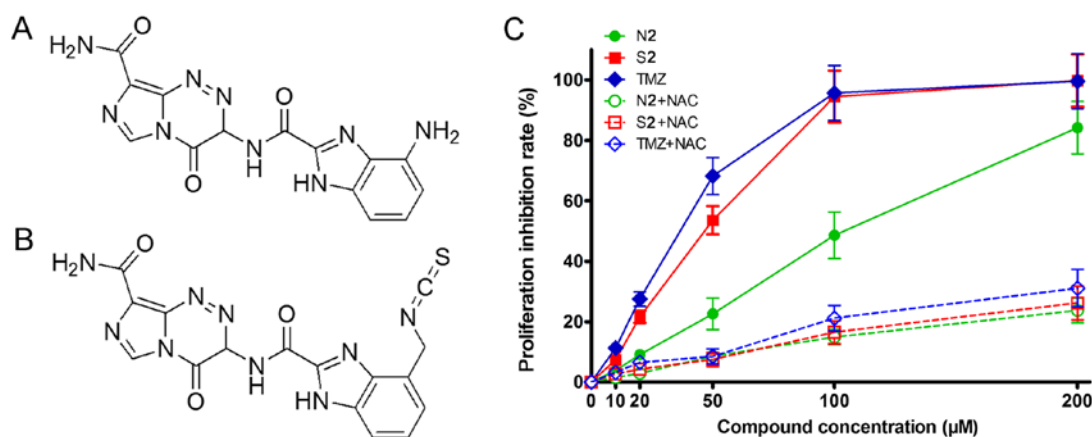


Figure 1. Structure and effect of carboxamide derivatives on the proliferation in glioma cells. Structures of (A) N2 and (B) S2. (C) MTS assay results revealed that the carboxamide derivatives and TMZ suppressed the proliferation of PC12, U251 and H4 cells. The proliferation inhibition curve shows that the carboxamide derivatives and TMZ exhibited inhibitory activity, and that NAC attenuated the activity of these compounds significantly.

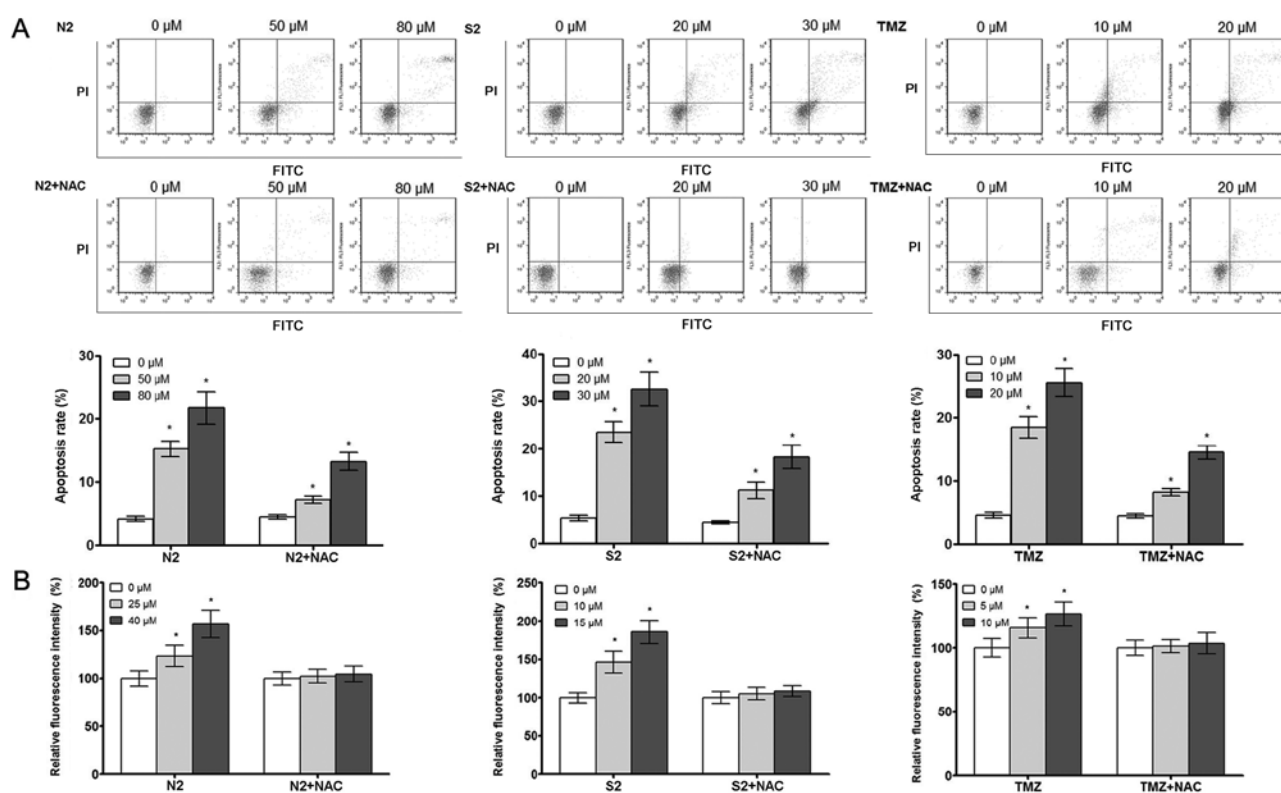


Figure 2. Effects of carboxamide derivatives and TMZ as inducers of apoptosis and oxidative damage in U251 cells. (A) Flow cytometry revealed that carboxamide derivatives (N2 and S2) and TMZ induced the apoptosis of U251 cells. NAC reduced the apoptosis induced by these compounds. (B) Fluorescence assay demonstrated that the carboxamide derivatives and TMZ induced the oxidative damage of U251 cells and NAC reversed the oxidative activity of the compounds. * $P < 0.05$ vs. 0 µM. NAC, N-Acetyl-L-cysteine; TMZ, temozolomide.

MTS assay also revealed that the inhibition of proliferation by S2 and N2 was significantly decreased by NAC in U251 cells, suggesting that the activity of the carboxamide derivatives may partly involve oxidative damage (Fig. 1C). The non-toxic doses (inhibition rate $< 10\%$) for N2 (25 µM), S2 (10 µM) and TMZ (5 µM), and low-toxicity doses (inhibition rate $< 15\%$) for N2 (40 µM), S2 (15 µM) and TMZ (10 µM), were taken forward to the following experiments, with the exception of the apoptosis assay, to avoid inhibiting cell proliferation. A 0 µM concentration was included as the control group.

Carboxamide derivatives are inducers of apoptosis and oxidative damage in U251 cells. The flow cytometry and ROS assay results show that the carboxamide derivatives induced apoptosis (Fig. 2A) and oxidative damage (Fig. 2B) in the U251 cells, indicating that the carboxamide derivatives may suppress the proliferation of U251 cells via these mechanisms. The ROS-inducing effects of N2 and S2 were eliminated by 20 µM NAC (Fig. 2B), further supporting the suggestion that oxidative damage may be an important mechanism for the antitumor activity of these compounds.

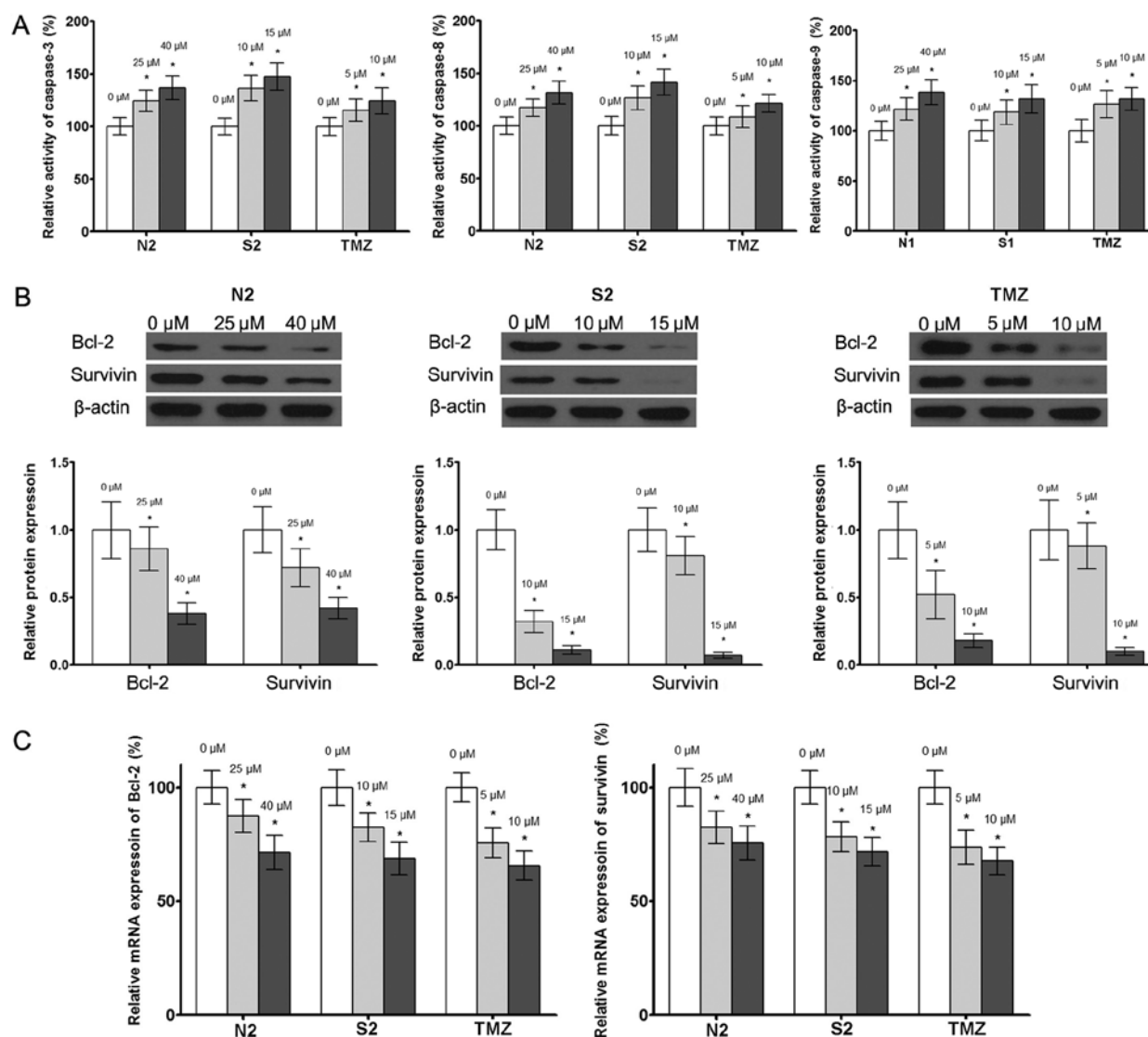


Figure 3. Effect of carboxamide derivatives and TMZ on the expression of apoptosis-associated genes in U251 cells. (A) Caspase activity assays showed that carboxamide derivatives (N2 and S2) and TMZ induced caspase-3, -8 and -9 activity in U251 cells. (B) Western blot assays demonstrated that the carboxamide derivatives and TMZ reduced Bcl-2 and survivin protein expression levels in U251 cells. (C) Carboxamide derivatives and TMZ also reduced the mRNA expression levels of Bcl-2 and survivin in U251 cells. * $P < 0.05$ vs. 0 μ M. TMZ, temozolomide.

Effects of carboxamide derivatives on caspase activity and apoptosis-associated mRNA in U251 cells. The results of the caspase-3, -8 and -9 activity assays demonstrated that the carboxamide derivatives were able to induce caspase-3, -8 and -9 activity (Fig. 3A). In addition, western blotting and RT-qPCR results indicated that the carboxamide derivatives reduced the protein (Fig. 3B) and mRNA (Fig. 3C) expression levels of Bcl-2 and survivin in U251 cells. This indicates that these carboxamide derivatives may induce the apoptosis of U251 cells by altering the activity of caspases-3, -8 and 9, and the expression of apoptosis-associated genes.

Discussion

TMZ is a common anti-glioma drug used worldwide; however, its toxicity is a limitation for clinical application (14,15). Therefore, the aim of the present study was to develop novel compounds with lower toxicity in order to reduce the side-effects of antitumor treatment.

The activity of two carboxamide derivatives as inhibitors of cell proliferation was investigated, and it was found that N2 in particular displayed reduced inhibitory activity compared with TMZ, suggesting the carboxamide derivatives may have potential as drugs with lower toxicity.

‘Survival with tumor’ is a novel tumor therapeutics concept in which the aim is not to kill all tumor cells, but to improve the quality of life of the patient and enable them to survive with the tumor via a less toxic treatment with fewer side effects (16). With this idea in mind, carboxamide derivative N2 and the structurally similar compound S2 were selected from two series of AGPS-targeting carboxamide derivatives, and their anti-glioma activity in the U251 cell line was measured. The mechanism of these carboxamide derivatives was also investigated.

The inhibitory effect of N2, S2 and TMZ on the proliferation of U251, H4 and PC12 cells was measured, and the results indicated that the inhibitory activity of N2 was markedly reduced compared with that of S2 and TMZ in all cell lines,

indicating its potential to be used for 'survival with tumor' treatment.

Apoptosis is an important mechanism of action of anti-tumor drugs (17). The mechanism underlying the effects of carboxamide derivatives on the proliferation of glioma cells was examined, and the flow cytometric quantification of TUNEL staining indicated that the carboxamide derivatives could induce the apoptosis of U251 cells. Therefore, it was speculated that apoptosis may be one of the mechanisms by which carboxamide derivatives suppress the growth of glioma cells. Bcl-2 and survivin are apoptosis resistance genes, and the expression of both is increased in tumor cells, which affects the activity of antitumor drugs with the inactivation of caspases (18,19). The present study demonstrated that Bcl-2 and survivin expression levels were suppressed, and the activities of caspase-3, -8 and -9 were increased in U251 cells following treatment with the carboxamide derivatives N2 and S2, indicating that the carboxamide derivatives induce apoptosis via the regulation of apoptosis-associated genes.

Oxidative damage is one of major mechanisms by which apoptosis is induced (20,21). The present study indicated that carboxamide derivatives induce the oxidative damage of U251 cells, and their oxidative activity was attenuated by the antioxidant NAC (22), indicating the effect of the compound partially involved the triggering of oxidative stress. Furthermore, it was observed that there was greater inhibition of proliferation and oxidative damage with compound S2 compared with N2. It is speculated that the stronger oxidative effect of S2 may be associated with the isothiocyanate group, which is reported to possess strong oxidation activity (23,24). The cell cycle also has an effect on cell proliferation, and further studies of the effects of carboxamide derivatives on the cell cycle remain to be performed. However, a limitation of the present study is that a rat cell line, not a human cell line, was used as the normal control.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

LH conceived and designed the study. JZ acquired the data. TY performed the data analysis.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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