Contents lists available at ScienceDirect

Translational Oncology

Original Research

The antitumor activity of a novel GCN2 inhibitor in head and neck squamous cell carcinoma cell lines

Jeongjae Lee [a,b], Bhumsuk Keam [a,c,*], Soyeon Kim [a,b], Jung-Nyoung Heo [d], Eunkyo Joung [e], Miso Kim [a,c], Tae Min Kim [a,c], Dong-Wan Kim [a,b,c], Dae Seog Heo [a,c]

[a] Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea
[b] Integrated Major in Innovative Medical Science, Seoul National University Graduate School, Seoul, Republic of Korea
[c] Department of Internal Medicine, Seoul National University Hospital, Seoul, Republic of Korea
[d] Therapeutics and Biotechnology Division, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea
[e] Clinical Science, Aston Sci. Inc., Seoul, Republic of Korea

ARTICLE INFO

ABSTRACT

Background: General control nonderepressible 2 (GCN2) senses amino acid deprivation and activates activating transcription factor 4 (ATF4), which regulates many adaptive genes. We evaluated the impact of AST-0513, a novel GCN2 inhibitor, on the GCN2-ATF4 pathway. Additionally, we evaluated the antitumor effects of AST-0513 in amino acid deprivation in head and neck squamous cell carcinoma (HNSCC) cell lines.
Methods: GCN2 expression in HNSCC patient tissues was measured by immunohistochemistry. Five HNSCC cell lines (SNU-1041, SNU-1066, SNU-1076, Detroit-562, FaDu) grown under amino acid deprivation conditions, were treated with AST-0513. After AST-0513 treatment, cell proliferation was measured by CCK-8 assay. Flow cytometry was used to evaluate apoptosis and cell cycle phase. In addition, immunoblotting was performed to evaluate the effect of AST-0513 on the GCN2-ATF4 pathway, cell cycle arrest, and apoptosis.
Results: We demonstrated that GCN2 was highly expressed in HNSCC patient tissues. AST-0513 inhibited the GCN2-ATF4 pathway in all five HNSCC cell lines. Inhibiting the GCN2-ATF4 pathway during amino acid deprivation reduced HNSCC cell proliferation and prevented adaptation to nutrient stress. Moreover, AST-0513 treatment led to p21 and Cyclin B1 accumulation and G2/M phase cycle arrest. Also, apoptosis was increased, consistent with increased bax expression, increased bcl-xL phosphorylation, and decreased bcl-2 expression.
Conclusion: A novel GCN2 inhibitor, AST-0513, inhibited the GCN2-ATF4 pathway and has antitumor activity that inhibits proliferation and promotes cell cycle arrest and apoptosis. Considering the high expression of GCN2 in HNSCC patients, these results suggest the potential role of GCN2 inhibitor for the treatment of HNSCC.

Introduction

The integrated stress response (ISR) signaling pathway allows eukaryotic cells to adapt to various cellular stresses. ISR is activated by sensor proteins that phosphorylate eIF2α when they recognize various stress stimuli, such as endoplasmic reticulum stress, nutrient deprivation and virus infection [1,2]. Following the ISR activation, global protein synthesis is reduced, but expression of specific genes that have two or more open reading frames in their 5′ untranslated region, including activating transcription factor 4 (ATF4), is increased [3,4]. ATF4 regulates the expression of many adaptive genes that promote tumor cell survival by maintaining cellular homeostasis [5]. Although the majority of ATF4-regulated genes maintain homeostasis, ATF4 also regulates the expression of genes that induce apoptosis. Consequently, ATF4 can promote apoptosis following persistent cellular stress [6,7]. Four main kinases function as sensor proteins of the ISR that sense different cellular stressors are heme-regulated eIF2α kinase (HRI), protein kinase R (PKR), protein kinase-like endoplasmic reticulum kinase (PERK), and general control nonderepressible 2 (GCN2) [1,2]. Among these, GCN2 senses amino acid deprivation by binding uncharged tRNA, and plays a major role in adaptation to amino acid deprivation [8–10].

Since tumor cells abnormally grow and proliferate, the cells of the tumor center are faced with nutrient deficiency, including a lack of glucose, oxygen, and amino acids, leading to metabolic stress. In

response to this metabolic stress, it is critical for tumor cells to maintain cellular homeostasis to survive and proliferate. GCN2 can be essential for tumor progression by maintaining amino acid homeostasis. Previous research has suggested the importance of the GCN2-ATF4 pathway for tumor cell survival and proliferation under amino acid deprivation [11]. GCN2 promotes tumor angiogenesis to help tumor cells evade the effects of nutrient stresses [12]. Additionally, GCN2 overexpression can be a predictive factor that has been correlated with poor prognosis in papillary renal cell carcinoma [13]. GCN2 controls c-myc expression, protecting tumor cells from c-myc-induced apoptosis and facilitating adaptation to nutrient stress [14]. GCN2 provides cytoprotective effect against vemurafenib in BRAF mutant cancer and causes tumor cells to become resistant to chemotherapies such as cisplatin [15] and L-asparaginase [16,17].

Targeting GCN2 might be an effective therapeutic strategy as this would prevent tumor cell adaptation to various stresses. Recently, there have been various attempts to synthesize new molecules that can inhibit the GCN2-ATF4 pathway; however, very few GCN2 inhibitors have been developed [17–19]. In this study, we demonstrated GCN2 expression in head and neck squamous cell carcinoma (HNSCC) patient tissues, and evaluated the effect of a novel GCN2 inhibitor, AST-0513, on the GCN2-ATF4 pathway. Furthermore, we investigated the antitumor effect of AST-0513 by evaluating proliferation, cell cycle progression, and apoptosis in HNSCC cells treated with AST-0513 under amino acid deprivation.

Materials and methods

Immunohistochemistry (IHC)

Patients diagnosed with locally advanced head and neck squamous cell carcinoma (HNSCC) and treated at Seoul National University Hospital from December 2004 to November 2012 were analyzed. Demographics, clinical, and pathologic data were obtained from medical records. All patients received radical surgery, and paraffin-embedded tumor samples were used for IHC. This study was approved by Institutional Review Boards of Seoul National University Hospital (approval number: H-1911-203-1088). Tissue microarray (TMA) was constructed for further immunohistochemical analysis. Subsequently, TMA blocks were sectioned and stained for GCN2. Anti-GCN2 antibody [EPR5970 (2)] (ab134053, Dawinbio Inc, Seoul, Korea) was used for IHC. GCN2 staining was scored semi-quantitatively based on the intensity of cytoplasmic staining using a 3-point scale from 0 to 3: (0: negative; 1: weak positive; 2: moderate positive; 3: strong positive).

Gene Expression Profiling Interactive Analysis (GEPIA) dataset analysis

GEPIA is a newly designed interactive web service that uses a standard processing pipeline to analyze the RNA sequencing expression data of 9,736 cancers and 8,587 normal tissue samples from the TCGA and GTEx studies. Tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, comparable gene finding, correlation analysis, and dimensionality reduction analysis are all customizable capabilities in GEPIA (http://gepia.cancer-pku.cn/) [20].

Cell lines and cell culture

Human HNSCC cell lines Detroit-562 and FaDu (purchased from the American type culture collection, Manassas, Virginia, USA) were cultured in Eagle's Minimum Essential Medium, and SNU-1041, SNU-1066, SNU-1076 (purchased from the Korean cell line bank, Seoul, Korea) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Billings, MT, USA). Each cell culture media was supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin (Gibco). To generate cellular stress through amino acid

deficiency, we used L-histidinol, a histidine analogue, which mimics histidine deprivation by blocking histidyl tRNA [10].

Reagents and antibodies

AST-0513, a novel GCN2 inhibitor, was provided by Aston Science and Korea Research Institute of Chemical Technology. AST-0513 was dissolved in DMSO. L-histidinol dihydrochloride (≥98%) (Sigma-Aldrich, St. Louis, Missouri, USA), used to generate amino acid deficiency, was dissolved in sterilized triple distilled water. Primary antibodies specific for GCN2 (#3302), eIF2α (#9722), phospho-eIF2α (Ser51) (#9721), ATF4 (D4B8) (#11815), Cyclin B (#12231), cdc25C (#4688), phospho-cdc25c (Ser216) (#4901), cdc2 (#77055), phospho-cdc2 (Tyr15) (#4539), bax (#2772), bcl-2 (#4223), bcl-xL (#2764), cleaved caspase 3 (Asp175) (#9661), and cleaved caspase 9 (Asp330) (#9501) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to phospho-GCN2 (Thr899) (#ab75836), phospho-bcl-xL (Ser62) (#21061), and β-actin (#A5441) were purchased from Abcam (Cambridge, CB, UK), Signalway Antibody (College Park, MD, USA) and Sigma-Aldrich, respectively. These antibodies were used for western blotting.

Quantitative real-time RT-PCR (qRT-PCR)

RNAs extraction from SNU-1041 cultured in four different conditions was conducted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instruction. Extracted RNAs were reverse transcribed into cDNA with SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The StepOnePlus Real-Time PCR system and Power SYBR green PCR Master Mix (Thermo fisher Scientific) were used to quantify gene expression. β-actin was used as endogenous control. Sequences of primers are listed in Supplementary Table S1.

siRNA transfection

SNU-1041 cells were transfected with ATF4 or negative control siRNA (BIONEER, Daejeon, Korea). Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) was used according to the manufacturer's instruction. Transfected cells were treated AST-0513 alone or with histidinol for 72 hours, and then cell proliferation assay was conducted.

Cell proliferation assay

HNSCC cell lines were seeded overnight at a density of 3×10^3 cells per well in 96-well plates, then cultured for 72 hours with various concentration of AST-0513 (0~5 μM) under normal condition or amino acid deficient condition. The optimal L-histidinol concentration for each cell line was used to generate the amino acid deficient condition. The Cell Counting Kit-8 (CCK-8) colorimetric assay (Dojindo, Rockvile, MD, USA) was used to analyze cell proliferation. Absorbance was measured at 450 nm in an Eon Microplate spectrophotometer (BioTek, Winooski, VT, USA).

Western blotting

Cells were resuspended in cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich), PMSF (Sigma-Aldrich), and PhosSTOP (Merck, Burlington, MA, USA) at 4°C for 20 minutes. The supernatant was collected after centrifuging at 13,000 rpm for 15 minutes at 4°C. Equal amounts of proteins were separated on a SDS-polyacrylamide gel (Thermo Fisher Scientific) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Next, the PVDF membrane was blocked with 5% skim milk for two hours at room temperature before being probed with diluted primary antibodies
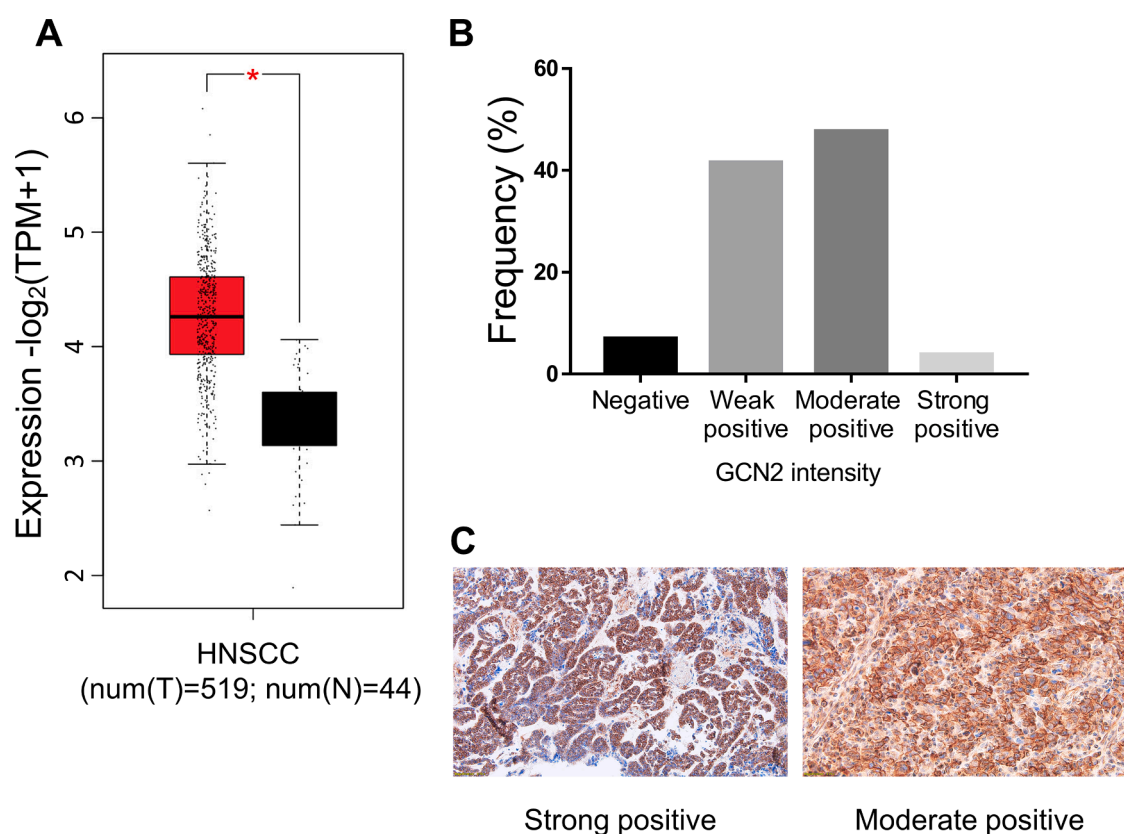
Fig. 1. GCN2 expression in HNSCC patient tissues. (A) The distribution of HNSCC tissues (red) and normal tissues (black) GCN2 mRNA expression. (B) The distribution of GCN2 IHC intensity. (C) Representative image of strong positive (left) and moderate positive (right). HNSCC, head and neck squamous cell carcinoma; T, tumor; N, normal.

at 4°C overnight and diluted secondary antibodies conjugated to HRP at room temperature for two hours. ECL detection reagent (GE Healthcare, Little Chalfont, BM, UK) was used to detect the signals, which were then visualized using the LAS 4000 mini (GE Healthcare). β-actin was used as a loading control.

Cell cycle analysis and apoptosis assays

Cells were seeded overnight at a density of 3×10^5 per well in 60 mm plates. Cells grown under an amino acid rich or deficient condition were treated with AST-0513 at different concentrations for 48 hours. Cells were harvested, washed twice with Dulbecco's phosphate-buffered saline (DPBS), and fixed with cold 75% ethanol at 4 °C overnight. Then, the cells were washed again with DPBS and incubated with RNase A for 20 minutes, followed by Propidium Iodide (PI) (BD Biosciences) staining for 1 hour at room temperature. Cell cycle analysis was performed on the FACS Calibur instrument. The data were analyzed by FlowJo v10.8.

Cells were seeded overnight at a density of 5×10^5 per well in 6-well plates. grown under an amino acid rich or deficient condition were treated with AST-0513 at different concentrations for 24 hours. After 24 hours, cells were stained with Annexin V- FITC (BD Biosciences, San Jose, California, USA) and PI (BD Biosciences), then the cells were analyzed by flow cytometry using a FACS Calibur instrument (BD Biosciences). The data were analyzed by FlowJo v10.8 (LCC, Ashland, OR, USA).

Statistical analysis

Data are represented as the mean ± standard deviation (SD). Data were analyzed by GraphPad Prism v7.0 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (one-way ANOVA) was

Table 1
Patient characteristics.

	Total $N = 130$	N (%)
Sex	Male	93 (71.5%)
	Female	37 (28.5%)
Location	Oropharynx	51 (39.2%)
	Hypopharynx	12 (9.2%)
	Larynx	8 (6.2%)
	Oral cavity	45 (34.6%)
	Nasal cavity	5 (3.8%)
	Others	9 (7.0%)
Stage	I	13 (10.0%)
	II	16 (12.3%)
	III	29 (22.3%)
	IV	71 (54.6%)
	Unknown	1 (0.8%)
GCN2 expression	Negative	9 (6.9%)
	Weak positive	54 (41.5%)
	Moderate positive	62 (47.7%)
	Strong positive	5 (3.8%)

performed to compare differences between multiple groups, and two-sided $P < 0.05$ was considered statistically significant.

Results

High GCN2 expression in HNSCC patient tumors

We used the GEPIA dataset to compare GCN2 mRNA expression in HNSCC tissues to normal tissues. The GCN2 mRNA expression level in HNSCC tissues was significantly higher than that of normal tissues (Fig. 1A). Additionally, we performed IHC to evaluate GCN2 expression in HNSCC patient tissues. Of the 130 patients included in this study, nine
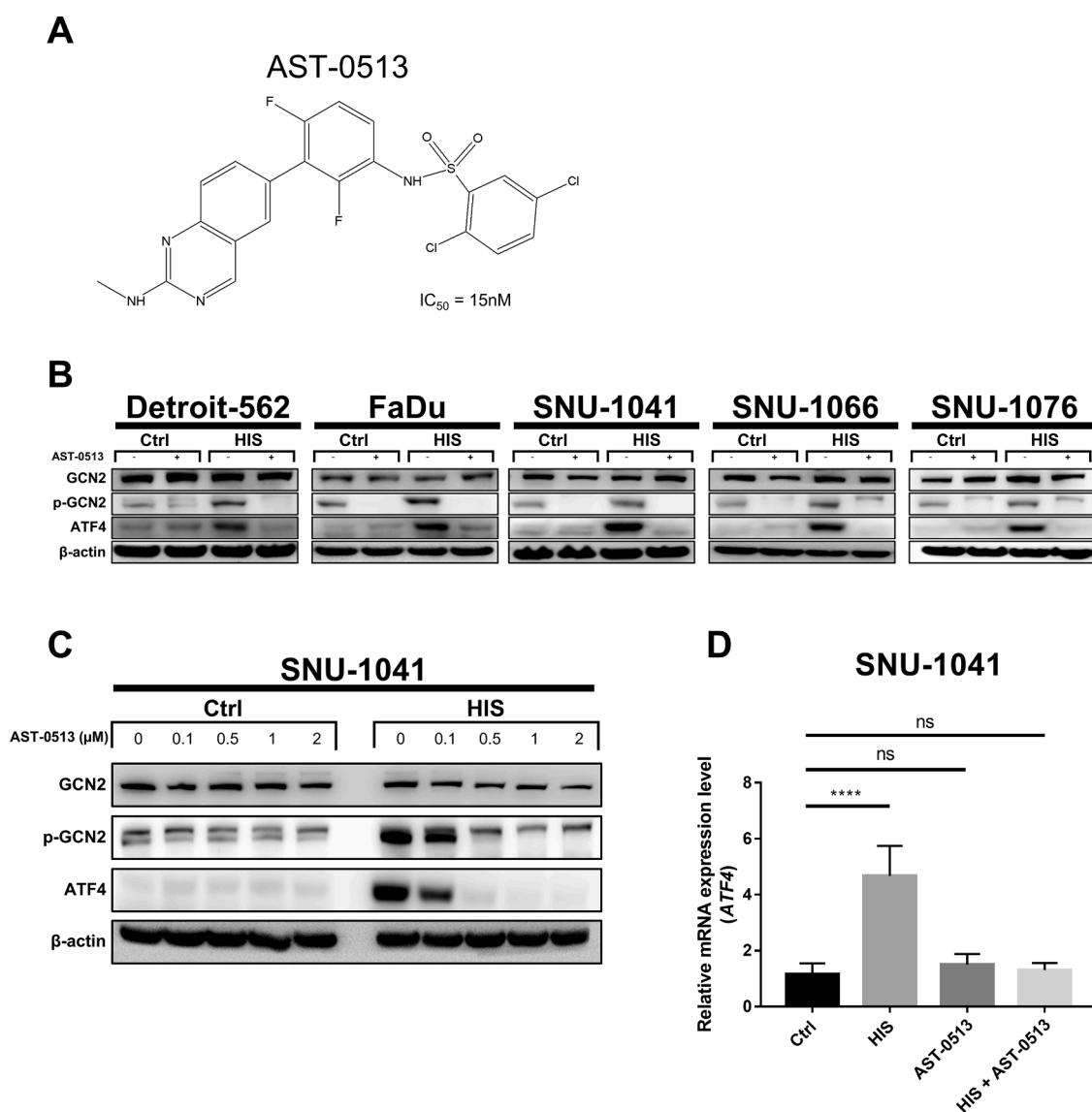
Fig. 2. AST-0513 inhibits the GCN2-ATF4 pathway in HNSCC cell lines under amino acid deprivation. (A) Structure and IC$_{50}$ value of AST-0513 (B) HNSCC cell lines were treated with 1 μM AST-0513 under normal condition or amino acid deficient condition for 6 hours. The lysates were examined by Western blotting. (C) SNU-1041 cells were treated with AST-0513 at the indicated concentration under normal condition or amino acid deficient condition for 6 hours. The lysates were examined by Western blotting. (D) Relative mRNA expression level of *ATF4* in different conditions examined by qRT-PCR. SNU-1041 cells were treated with 1 μM AST-0513 under normal condition or amino acid deficient condition for 4 hours. All data are shown as mean±SD. ****$P < 0.0001$. Statistical significance was determined by one-way ANOVA. Ctrl, control; HIS, L-histidinol; p, phosphorylated; ns, not significant.

patients (6.9%) were GCN2 negative, and 67 patients (51.5%) had GCN2 intensity of moderate or strong positive (Table 1, Fig. 1B). IHC showed representative images of GCN2 positive tumors (Fig. 1C). On the other hand, disease-free survival and overall survival were not different based on GCN2 positivity (data not shown). These results demonstrated that GCN2 expression levels are high in the majority of HNSCC tumors.

AST-0513 inhibits the GCN2-ATF4 pathway in HNSCC cells

We generated a novel GCN2 kinase inhibitor, AST-0513. AST-0513 showed 97% inhibition at 1 uM and the IC$_{50}$ value was 15 nmol/L (Fig. 2A). To evaluate whether AST-0513 inhibits the GCN2-ATF4 pathway in HNSCC cells grown under amino acid deprivation, we assessed p-GCN2 and ATF4 expression after AST-0513 treatment. AST-0513 treatment inhibited the GCN2-ATF4 pathway in all HNSCC cell examined (Fig. 2B). Furthermore, we observed that AST-0513 inhibited p-GCN2 and ATF4 in HNSCC cells grown under amino deprivation in a

dose-dependent manner (Fig. 2C). To confirm that AST-0513 blocks the GCN2/ATF4 pathway at the mRNA level, qRT-PCR was performed. We observed that mRNA expression of *ATF4* also decreased by AST-0513 in amino acid starvation (Fig. 2D).

AST-0513 inhibits HNSCC cell proliferation by blocking GCN2-ATF4 pathway and prevents adaptation to amino acid deprivation

To evaluate the anti-proliferation effect of AST-0513 on HNSCC cells, we performed CCK-8 cell viability assay. Because the susceptibility to stress was different for each cell line, each HNSCC cell line was treated with an appropriate concentration of L-histidinol to generate the amino acid deprivation condition. Under normal cell culture conditions, except for high concentration of AST-0513, there was almost no anti-proliferation effect. On the other hand, in amino acid deprivation condition, AST-0513 showed a significant anti-proliferation effect from a low concentration in the amino acid deprivation cell cultures. All cell
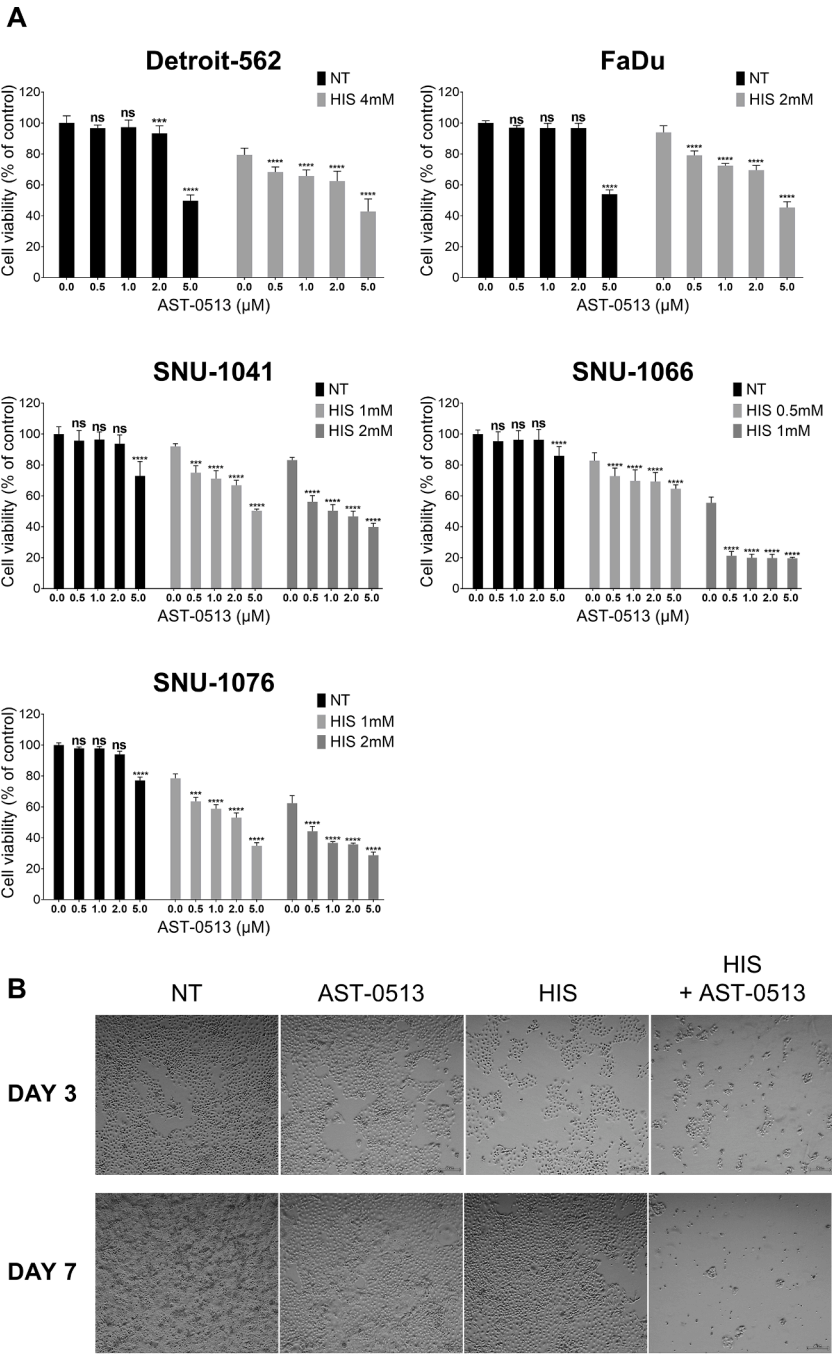
A

Fig. 3. The anti-proliferative effect of AST-0513 on HNSCC cell lines under amino acid deprivation. (A) HNSCC cell lines were treated with various concentration of AST-0513 (0.5~5 µM) under normal condition (NT) or amino acid deficient condition (HIS) for 72 hours. CCK-8 assay was performed to assess HNSCC cell proliferation. (B) SNU-1041 cells were treated with 2 µM AST-0513 under normal condition (NT) or amino acid deficient condition (L-histidinol) as indicated, and light microscopy images of SNU-1041 cells were taken at 40x magnification, at day 3 and 7 after treatment. (C) SNU-1041 cells were treated with 2 mM L-histidinol and/or 2 µM AST-0513, then counted at day 3 and 7 after treatment. Media was changed at day 3. All data are shown as mean±SD. ***$P <$ 0.001, ****$P <$ 0.0001. Statistical significance was determined by one-way ANOVA. NT, no treatment; HIS, L-histidinol; ns, not significant.
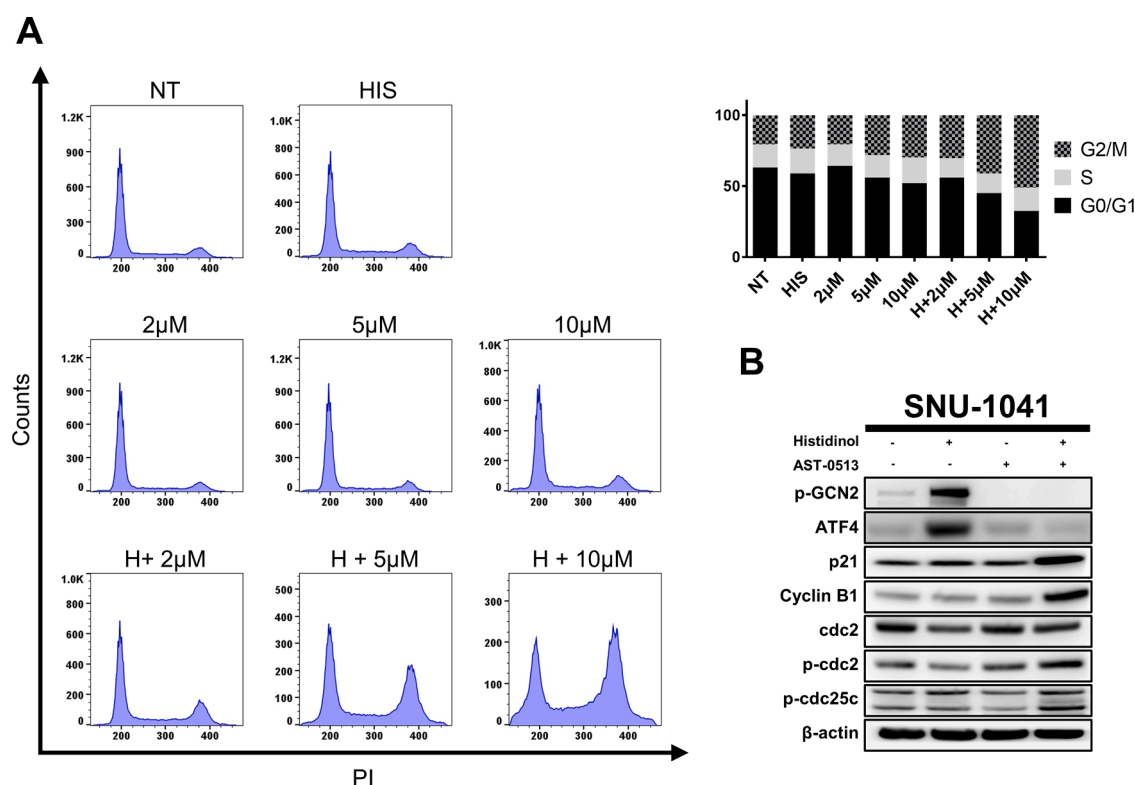
B

C
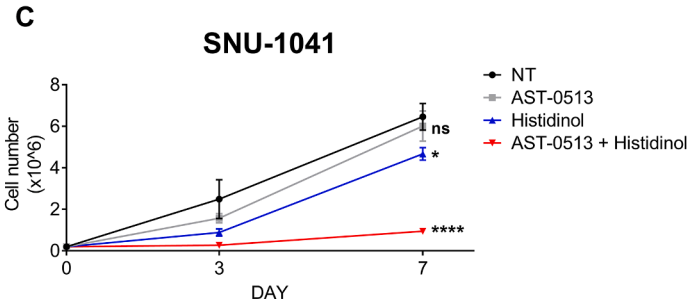
Fig. 4. AST-0513 induces HNSCC cell lines to G2/M arrest under amino acid deprivation. (A) SNU-1041 cells were treated with elevated concentration (2, 5, 10 μM) of AST-0513 and/or 2 mM L-histidinol for 48 hours. After incubation, fixation and PI staining were performed. The distribution of each phase of cell cycle was assessed by flow cytometry. (B) SNU-1041 cells were treated with 2 μM AST-0513 and/or 2 mM histidinol for 24 hours. The expression of cell cycle-regulated proteins was examined by Western blotting. NT, no treatment; HIS, L-histidinol; p, phosphorylated.

lines showed the same pattern, but exhibited difference in sensitivity to AST-0513 according to cell lines and L-histidinol concentration (Fig. 3A). Next, to confirm cellular effects of AST-0513 is dependent on blocking GCN2/ATF4 pathway. We performed Knockdown (KD) of ATF4 gene. ATF4 expression was weakly expressed after 48 hours of KD, but proliferation inhibition occurred significantly in the treatment of L-histidinol alone. In addition, unlike the control group, further inhibition due to AST-0513 did not occur from the concentration over 0.5 μM (Supplementary Fig. S1). Therefore, we confirmed that the cellular effect of AST-0513 is dependent on the blocking of the GCN2/ATF4 pathway. Additionally, we performed a 7-day proliferation assay. Although proliferation was inhibited by amino acid deprivation up to day 3, proliferation was restored on day 7. In contrast, amino acid deprived cells treated with AST-0513 still remained anti-proliferative on day 7 (Fig. 3B, C).

AST-0513 induces G2/M phase cell cycle arrest in amino acid deprived HNSCC cells

Given the interdependence of proliferation and cell cycle progression, we performed cell cycle analysis. AST-0513 increased the number of G2/M phase cells and decreased the number of G0/G1 phase cells in a dose-dependent manner (Fig. 4A). To expand on these results, we performed Western blotting of cell cycle regulatory proteins. Consistent with the result of cell cycle analysis, the expression of p21, Cyclin B1, phospho-cdc2, and phospho-cdc25c indicated that G2/M phase cell cycle arrest was increased following AST-0513 treatment under amino acid deprivation (Fig. 4B).

AST-0513 induces a increase of apoptosis in HNSCC cell lines under amino acid deprivation

We investigated whether AST-0513 affects apoptosis during cellular stress due to amino acid deprivation. The number of cells with apoptotic morphology was not significantly changed following single treatment of AST-0513 or L-histidinol, but apoptosis relatively increased when treated in combination (Fig. 5A). Flow cytometry analysis followed after Annexin V-FITC/PI staining. When AST-0513 or L-histidinol was treated alone, there was a slight increase in apoptotic cells, and AST-0513 treatment under L-histidinol stress increased apoptosis to a significant level., unlike other conditions (Fig. 5B). We performed Western blotting of apoptosis-related proteins. Following AST-0513 treatment of cell under L-histidinol stress, the expression of pro-apoptotic proteins, including cleaved PARP, cleaved caspase-3, cleaved caspase-9, and bax were increased. On the other hand, anti-apoptotic protein bcl-2 was decreased, and phosphorylation of bcl-xL was increased (Fig. 5C).

Discussion

In this study, we demonstrated that GCN2 is expressed in HNSCC and AST-0513 blocked the GCN2-ATF4 pathway in amino acid deprived HNSCC cells. Our data also showed that AST-0513 inhibited cell proliferation in HNSCC cells under nutrient stress and had antitumor activity that caused cell cycle arrest and apoptosis.

Since AST-0513, a novel GCN2 inhibitor, is newly synthesized, there is not much data. However, metabolic stability and kinetic solubility of AST-0513 were confirmed (Supplementary Table S2, S3), and adverse effects on organs were not observed in mice. As the study of AST-0513 progresses, more data be expected to accumulate. In all the HNSCC cell lines used in the experiment, AST-0513 treatment of cell under amino acid deprivation blocked the GCN2-ATF4 pathway and inhibited
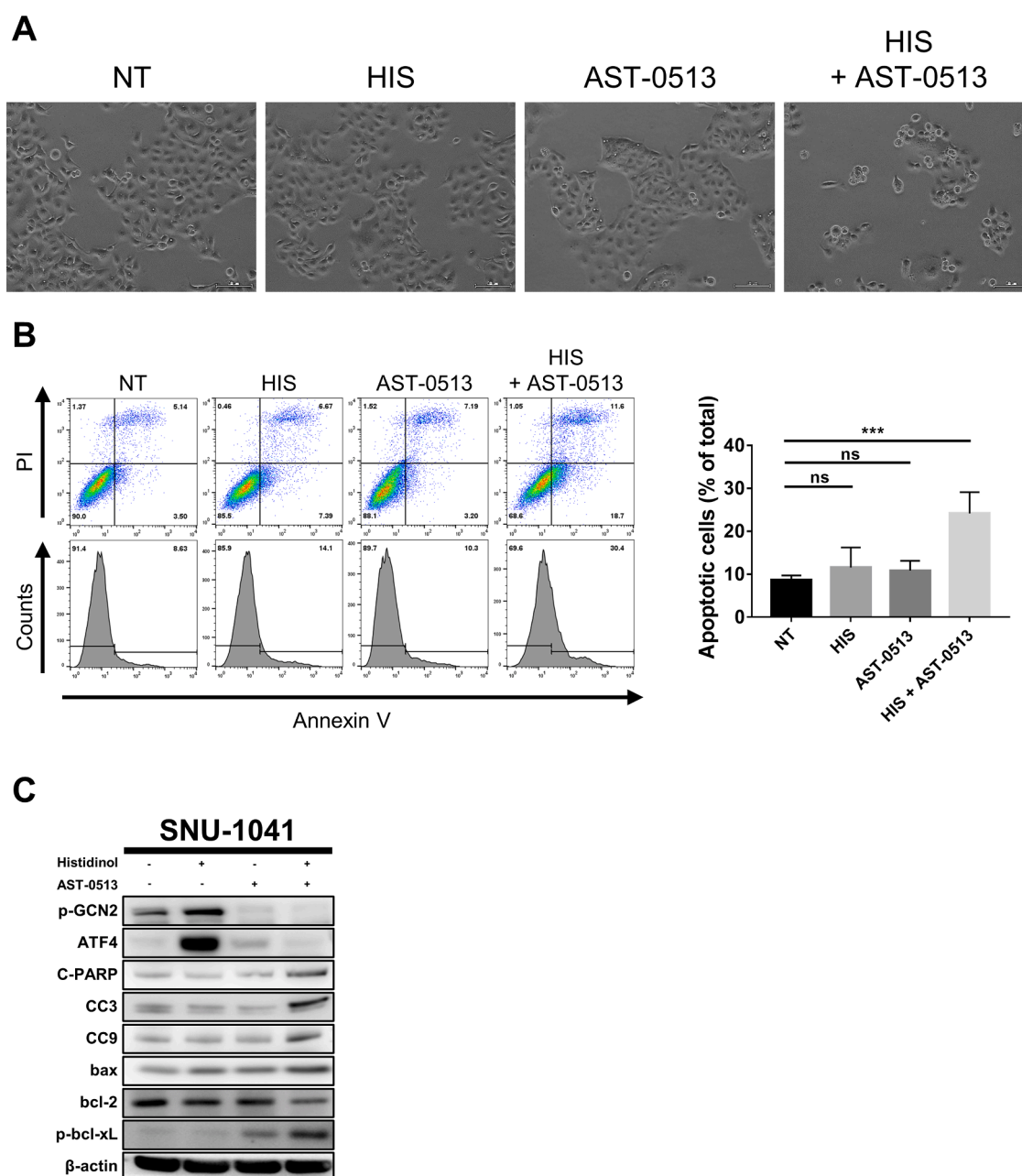
Fig. 5. AST-0513 induces an increase of apoptosis in amino acid deprived HNSCC cells (A). SNU-1041 cells were treated with 2 μM AST-0513 and/or 2 mM histidinol for 24 hours. After incubation, light microscopy images of SNU-1041 cells were taken at 100x magnification. (B) Cells were stained by Annexin V-FITC/PI antibodies and analyzed by flow cytometry. (C) SNU-1041 cells were treated with 2 μM AST-0513 and/or 2 mM histidinol for 24 hours. The expression of apoptosis-related proteins was examined by Western blotting. The percentage of apoptotic cells is shown as mean±S.D. ***$P < 0.001$. Statistical significance compared with NT and other groups was determined by one-way ANOVA. NT, no treatment; HIS, L-histidinol; ns, not significant; p, phosphorylated; C-PARP, cleaved PARP; CC3, cleaved caspase 3; CC9, cleaved caspase 9.

cell proliferation. Under amino acid deprivation, HNSCC cell proliferation was restored on day 7, but AST-0513 treatment had a sustained antiproliferative effect. This suggests GCN2 plays a role in adaptation to amino acid starvation, whereas treatment with AST-0513 continued to suppress proliferation. The efficacy of AST-0513 increased when the cells were treated with a higher concentration of L-histidinol. Hence, GCN2 may be essential to cells in a high nutrient stress environment, indicating GCN2 inhibition could be effective in tumor types where nutrient stress occurs more frequently. Tumor cell sensitivity to L-histidinol and AST-0513 was different for each HNSCC cell line. The nutrient stress-sensitive cell lines, in which proliferation was significantly reduced even at very low L-hisitidinol concentrations, seemed to

be more sensitive to AST-0513.

Cell cycle arrest occurs during late G1 and late G2 checkpoints, stopping proliferation when DNA is damaged, and giving cells time to recover. However, if the cell cycle arrest persists, it can induce cell apoptosis [21]. Activation of cdc2/Cyclin B1 complex is important for the transition of the G2/M phase [22]; however, abnormal accumulation of Cyclin B1 has sometimes been observed during G2/M arrest [23,24]. According to our western blotting results, AST-0513 caused G2/M arrest in amino acid deprived HNSCC cells as AST-0513 induced Cyclin B1 accumulation. In addition, phosphorylation of cdc25c, which dephosphorylates and activates cdc2 [25], phospho-cdc2, and p21, a potent inhibitor of cdc2/Cyclin B1 complex [26], was increased.

Although previous studies have shown GCN2 increases p21 expression during amino acid deprivation [27,28], p21 expression further increased when GCN2 was inhibited by AST-0513. Given GCN2 facilitates cancer cell adaptation to the stress environment by maintaining amino acid homeostasis, it is speculated that GCN2 inhibition leads to further DNA damage accumulation, resulting in persistent G2/M arrest that causes cell death. Though the exact mechanism of this phenomenon should be further investigated, these results provide evidence that AST-0513 treatment under amino acid deprivation induces G2/M arrest.

Apoptosis is a promising target for future anticancer therapy, and effective elimination of cancer cells through apoptosis is a major mechanism of several existing anticancer drugs [29]. Apoptosis is initiated by caspase activation and regulated by the Bcl-2 protein family. Among the Bcl-2 protein family, bax and bak act as proapoptotic effectors, whereas bcl-2 and bcl-xL act as antiapoptotic effectors [30]. In our study, following AST-0513 treatment of amino acid deprived HNSCC cell lines, the activation of caspase-3, caspase-9, and PARP was confirmed by the increased levels of each cleaved form. Furthermore, an increase of bax and a decrease of bcl-2 occurred. Phosphorylation of bcl-xL was also increased, which is thought to decrease the antiapoptotic function of bcl-xL. These results indicate AST-0513 promotes apoptosis upon amino acid deprivation. However, further studies are needed to demonstrate the specific pathways that induce apoptosis.

There are several limitations of our study. Although GCN2 expression was high in HNSCC, it is unclear whether GCN2 could represent a biomarker to determine the efficacy of AST-0513 treatment. Additionally, the artificially generated amino acid deficient condition *in vitro* may be different from the *in vivo* environment. Thus, studies that focus on identifying a biomarker that determines the efficacy of GCN2 inhibitor and *in vivo* studies are needed. Studies on possible adverse effects are necessary because GCN2 acts by the same mechanism in normal tissues. Despite these limitations, our study demonstrated the novel GCN2 inhibitor has antitumor activity on HNSCC *in vitro* suggesting there is a potential to treat HNSCC with the GCN2 inhibitor.

In conclusion, a novel GCN2 inhibitor, AST-0513, blocked the GCN2-ATF4 pathway, inhibited proliferation, and induced cell cycle arrest and apoptosis in HNSCC cells under amino acid deprivation. These results suggest the potential role of GCN2 inhibitor in the treatment of HNSCC with high GCN2 expression.

Conception and design: JL and BK. Development of methodology: JL, BK, SK, EJ and TMK. Acquisition of data (eg, provided cells, provided reagents and provided facilities, etc.): JL, J-NH, EJ and SK. Analysis and interpretation of data: JL, BK, SK, EJ and TMK, Study supervision: BK, SK, MK, TMK, D-WK and DSH, Writing, review and/or revision of the manuscript: all authors

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101592.

References

[1] K. Pakos-Zebrucka, I. Koryga, et al., The integrated stress response, EMBO Rep. 17 (10) (2016) 1374–1395.

[2] X. Tian, S. Zhang, et al., Targeting the integrated stress response in cancer therapy, Front. Pharmacol. 12 (2021), 747837.

[3] R.J. Jackson, C.U.T. Hellen, et al., The mechanism of eukaryotic translation initiation and principles of its regulation, Nat. Rev. Mol. Cell Biol. 11 (2) (2010) 113–127.

[4] D.E. Andreev, P.B. O'Connor, et al., Translation of 5′ leaders is pervasive in genes resistant to eIF2 repression, Elife 4 (2015) e03971.

[5] I.M.N. Wortel, L.T. van der Meer, et al., Surviving stress: modulation of ATF4-mediated Stress responses in normal and malignant cells, Trends Endocrinol. Metab. 28 (11) (2017) 794–806.

[6] R. Iurlaro, F. Püschel, et al., Glucose deprivation induces ATF4-mediated apoptosis through TRAIL death receptors, Mol. Cell. Biol. 37 (10) (2017).

[7] Y. Zong, S. Feng, et al., Up-regulated ATF4 expression increases cell sensitivity to apoptosis in response to radiation, Cell. Physiol. Biochem. 41 (2) (2017) 784–794.

[8] S.A. Wek, S. Zhu, et al., The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids, Mol. Cell. Biol. 15 (8) (1995) 4497–4506.

[9] J. Dong, H. Qiu, et al., Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain, Mol. Cell 6 (2) (2000) 269–279.

[10] P. Zhang, B.C. McGrath, et al., The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice, Mol. Cell. Biol. 22 (19) (2002) 6681–6688.

[11] J. Ye, M. Kumanova, et al., The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation, EMBO J. 29 (12) (2010) 2082–2096.

[12] Y. Wang, Y. Ning, et al., Amino acid deprivation promotes tumor angiogenesis through the GCN2/ATF4 pathway, Neoplasia 15 (8) (2013) 989–997.

[13] L. Ge, W. Chen, et al., GCN2 is a potential prognostic biomarker for human papillary renal cell carcinoma, Cancer Biomark. 22 (3) (2018) 395–403.

[14] S. Schmidt, D. Gay, et al., A MYC–GCN2–eIF2α negative feedback loop limits protein synthesis to prevent MYC-dependent apoptosis in colorectal cancer, Nat. Cell Biol. 21 (11) (2019) 1413–1424.

[15] S.-F. Wang, M-S. Chen, et al., Mitochondrial dysfunction enhances cisplatin resistance in human gastric cancer cells via the ROS-activated GCN2-eIF2α-ATF4-xCT pathway, OncoTargets Ther. 7 (45) (2016) 74132–74151.

[16] P. Bunpo, A. Dudley, et al., GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with the anti-cancer agent L-asparaginase, J. Biol. Chem. 284 (47) (2009) 32742–32749.

[17] A. Nakamura, T. Nambu, et al., Inhibition of GCN2 sensitizes ASNS-low cancer cells to asparaginase by disrupting the amino acid response, Proc. Natl. Acad. Sci. 115 (33) (2018) E7776.

[18] Y. Kato, K. Kunimasa, et al., GZD824 inhibits GCN2 and sensitizes cancer cells to amino acid starvation stress, Mol. Pharmacol. 98 (6) (2020) 669–676.

[19] J. Fujimoto, O. Kurasawa, et al., Identification of novel, potent, and orally available GCN2 inhibitors with Type I half binding mode, ACS Med. Chem. Lett. 10 (10) (2019) 1498–1503.

[20] Z. Tang, C. Li, et al., GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids. Res. 45 (W1) (2017) W98–w102.

[21] K. Vermeulen, D.R. Van Bockstaele, et al., The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer, Cell Prolif. 36 (3) (2003) 131–149.

[22] G.R. Stark, W.R. Taylor, Analyzing the G2/M checkpoint, Methods Mol. Biol. 280 (2004) 51–82.

[23] D. Tamura, T. Arao, et al., Bortezomib potentially inhibits cellular growth of vascular endothelial cells through suppression of G2/M transition, Cancer Sci. 101 (6) (2010) 1403–1408.

[24] H. Wang, T. Zhang, et al., Erianin induces G2/M-phase arrest, apoptosis, and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells *in vitro* and *in vivo*, Cell Death. Dis. 7 (6) (2016) e2247. -e.

[25] K. Liu, M. Zheng, et al., The role of CDC25C in cell cycle regulation and clinical cancer therapy: a systematic review, Cancer Cell Int. 20 (1) (2020) 213.

[26] T. Abbas, A. Dutta, p21 in cancer: intricate networks and multiple activities, Nat. Rev. Cancer 9 (6) (2009) 400–414.

[27] K.A. Manu, P.H. Cao, et al., p21cip1/waf1 coordinates autophagy, proliferation and apoptosis in response to metabolic stress, Cancers 11 (8) (2019).

[28] S.L. Lehman, G.J. Cerniglia, et al., Translational Upregulation of an Individual p21Cip1 Transcript Variant by GCN2 Regulates Cell Proliferation and Survival under Nutrient Stress, PLos Genet. 11 (6) (2015), e1005212.

[29] W. An, H. Lai, et al., Apoptotic pathway as the therapeutic target for anticancer traditional Chinese medicines, Front. Pharmacol. 10 (2019).

[30] C.F.A. Warren, M.W. Wong-Brown, et al., BCL-2 family isoforms in apoptosis and cancer, Cell Death. Dis. 10 (3) (2019) 177.