INTRODUCTION

Gastric adenocarcinoma is a cancer originating from glandular epithelium of the gastric mucosa and accounts for approximately 90-95% of total gastric cancer incidence (Smyth et al., 2020). Given the oligosymptomatic course of early stage of gastric cancer, about two-thirds of the patients are diagnosed as advanced stage, which can only be treated with chemo or radiotherapy (Zhao et al., 2017). Moreover, the curative potential of the current standard treatment continues to be unsatisfactory, despite the existence of multimodal therapeutic approaches (Stock and Otto, 2005). Drug resistance is still a common problem for gastric cancer treatment, and there is a huge demand to discover and explore a new therapeutic approach or novel drug.

Recent studies showed that targeting autophagy is a promising approach for chemotherapy (Dalby et al., 2010; Steeves et al., 2010; Fulda, 2018; Amaravadi et al., 2019; Acevo-Rodríguez et al., 2020). It is a multi-step mechanism, generally divided into five; (1) initiation, (2) elongation, (3) closure, (4) fusion and (5) degradation, orchestrated by a variety of autophagy related proteins. This process is essentially a cellular recycling program. Dysfunctional or damaged cellular components are sequestered by double-membraned vesicles called autophagosomes and brought into the lumen of lysosomes, where they are broken down into basic components such as amino acids.

Abstract

Gastric adenocarcinoma is among the top causes of cancer-related death and is one of the most commonly diagnosed carcinomas worldwide. Benzyl isothiocyanate (BITC) has been reported to inhibit the gastric cancer metastasis. In our previous study, BITC induced apoptosis in AGS cells. The purpose of the present study was to investigate the effect of BITC on autophagy mechanism in AGS cells. First, the AGS cells were treated with 5, 10, or 15 µM BITC for 24 h, followed by an analysis of the autophagy mechanism. The expression level of autophagy proteins involved in different steps of autophagy, such as LC3B, p62/SQSTM1, Atg5-Atg12, Beclin1, p-mTOR/mTOR ratio, and class III PI3K was measured in the BITC-treated cells. Lysosomal function was investigated using cathepsin activity and Bafilomycin A1, an autophagy degradation stage inhibitor. Methods including qPCR, western blotting, and immunocytochemistry were employed to detect the protein expression levels. Acridine orange staining and omnicathepsin assay were conducted to analyze the lysosomal function. siRNA transfection was performed to knock down the LC3B gene. BITC reduced the level of autophagy protein such as Beclin 1, class III PI3K, and Atg5-Atg12. BITC also induced lysosomal dysfunction which was shown as reducing cathepsin activity, protein level of cathepsin, and enlargement of acidic vesicle. Overall, the results showed that the BITC-induced AGS cell death mechanism also comprises the inhibition of the cytoprotective autophagy at both initiation and degradation steps.

Key Words: Benzyl isothiocyanate, AGS cell, Apoptosis, Autophagy, Lysosomal dysfunction
acids that can be returned to the cytosol and reused in order to keep the cells alive (Parzych and Klionsky, 2014; Yim and Mizushima, 2020).

Studies have reported that autophagy is involved in the regulatory process of cancer cells (Feng et al., 2014; Amaravadi et al., 2019). Cancer cells grow through constant stress conditions within the tumor microenvironment. Once an anti-cancer therapy is applied, it amplifies the stress signaling in cancer cells, which then activate autophagy (Amaravadi et al., 2019). Depending on the level of stress conditions, autophagy in cancer cells has a dual nature as either tumor suppressing signal or tumor promoting signal (Singh et al., 2018). Some studies have reported that the cytoprotective function of autophagy enables tumor cell survival, which can facilitate tumor progression and confer resistance to anticancer treatments (Guo et al., 2013; Li et al., 2017, 2019). Some studies have demonstrated that certain chemotherapeutics or molecularly targeted therapeutics can lead to the autophagic death of cancer cells (Mathew et al., 2007; Wong et al., 2017; Law et al., 2019). Accordingly, cytoprotective autophagy in cancers has become a key potential target for anti-cancer therapy, and the induction of excessive or sustained autophagy has been considered as an alternative strategy for eliminating tumors (Steeves et al., 2010; Claerhout et al., 2011).

Benzyl isothiocyanate (BITC) is an isothiocyanate compound found in plants of the mustard family, including mustard, radish, broccoli, cabbage, water cress, etc. (Moy et al., 2009; Singh and Singh, 2012). BITC was known to have a variety of pharmacological effects. BITC has anti-inflammatory effect through suppression of NF-κB, ERK 1/2, and Akt (Lee et al., 2009). BITC also reduced hyperglycemia by regulating oxidative stress and GLUT4 (Chuang et al., 2020). BITC was shown to have a broad-spectrum antibacterial effect (Li et al., 2021). BITC is also well known to have anticancer activity against cancer. Several studies have described the anti-cancer effects of BITC in a variety of cancer cells, including the inhibition of metastasis in colon cancer (Lai et al., 2010), promoting apoptosis in oral cancer (Yeh et al., 2016), inhibition of PI3K/AKT/FOXO pathway in pancreatic cancer (Boreddy et al., 2011), inducing apoptosis and cell cycle arrest in brain cancer (Zhu et al., 2013; Tang et al., 2016; Ma et al., 2018), inhibition of metastasis in melanoma (Lai et al., 2017) and more. In addition to its apoptotic, cell cycle arrest and anti-metastatic properties, BITC also influences the autophagy mechanism. In lung cancer cells and prostate cancer cells, it was reported that BITC induced the cytoprotective autophagy, but in breast cancer cell line, BITC induced autophagic cell death (Xiao et al., 2012; Lin et al., 2013; Zhang et al., 2017). The role of autophagy induced by BITC varies depending on the type and condition of cancer cells.

As in other cancers, BITC also has a chemotherapeutic effect on gastric cancer. Clinical evidence from China has demonstrated that isothiocyanates can protect against gastric cancer (Moy et al., 2009). BITC demonstrated its ability to inhibit the migration and invasion of human gastric adenocarcinoma AGS cells by suppressing ERK pathways (Ho et al., 2011). In our previous study, BITC induced apoptosis in AGS cells via reactive oxygen species-initiated mitochondrial dysfunction and DR4 and DR5 death receptor activation (Han et al., 2019). We hypothesized that BITC may also impact the autophagy mechanism of the AGS cells in addition to its apoptotic effect. Therefore, the present study was conducted to investigate the effect of BITC on the autophagy mechanism in AGS cells.

MATERIALS AND METHODS

Materials and reagents
All of the basic cell culture reagents were purchased from Invitrogen (Grand Island, NY, USA), Corning Inc. (NY, USA), and Welgene Inc (Daegu, Korea). To prepare the cellular extracts, RIPA lysis and extraction buffer were purchased from Thermofisher Scientific (Waltham, MA, USA), and protease and phosphatase inhibitors were purchased from Sigma Chemical Co (St. Louis, MO, USA). For cell viability assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were also purchased from Sigma Chemical Co. Antibodies against LC3B, Atg5, BECLIN1, p62/SQSTM1, PI3K class III, mTOR, p-mTOR, cathepsin B, and cathepsin D were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-cathepsin L, anti-LAMP2, anti-GAPDH, and anti-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). Anti-rabbit IgG (H+L), F(ab’2) fragment (Alexa Fluor® 488 conjugate), normal goat serum, LC3B siRNA II, and control siRNA were purchased from Cell Signaling Technology. Lipofectamine RNAiMAX reagent and Opti-MEM reduced serum medium were purchased from Thermo Fisher Scientific. All western blotting reagents were purchased from ECLPIS-BIOTECH (Daejeon, Korea) and Bio-Rad (Hercules, CA, USA). PVDF membranes were purchased from Koma Biotech (Seoul, Korea) and NC membranes were purchased from Bio-Rad. X-ray films were purchased from Thermofisher Scientific. PCR primers for hLC3B, hSQSM1 and beta-actin were purchased from BIONEER (Daejeon, Korea). Nucleolozol reagent was purchased from MACHEREY-NAGEL (Düren, North Rhine-Westphalia, Germany). The ReverTra Ace qPCR RT Master Mix kit was purchased from TOYOBO (Osaka, Japan), and the IQ™ SYBR® Green Supermix was obtained from Bio-Rad. Benzyl isothiocyanate (BITC), a pan-caspase inhibitor, Z-VAD-FMK, and the PI3K inhibitor, wortmannin were purchased from Selleckchem (Houston, TX, USA). Bafilomycin A1 was purchased from TOCRIS Bioscience (Bristol, UK). Omnicathepsin fluorogenic substrate (Z-FR-AMC) was purchased from ENZO Life Sciences (Farmingdale, NY, USA). 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Roche (Mannheim, Baden-Württemberg, Germany) and acridine orange (AO) was purchased from Sigma Chemical Co.

Cell culture and morphological observation
The human gastric cancer cell line, AGS (KCLB 21739), was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI-1640 medium (Welgene Inc.) containing 10% FBS and 5% antibiotics, which consisted of 1% penicillin-streptomycin and 0.1% amphotericin B. The culture was kept at 37°C in a humidified atmosphere with 5% CO2 and 95% air. Cells were seeded as a monolayer in a petri dish in a 1:10 ratio of cells to total media. They were passaged every time 90% confluence was reached.
For treatment, the cells were seeded to 70-80% confluency and incubated with different concentrations of BITC (5, 10, or 15 µM) for a specified duration. The changes in cellular morphology were observed using a fluorescence microscope (Leica, Wetzlar, Germany).

**Measurement of cell viability**

To determine the cell viability, an MTT assay was performed. AGS cells were plated at a density of 1×10^5 cells/well in 24-well plates overnight in RPMI-1640 medium supplemented with 10% FBS and 5% antibiotics. The next day, the cells were treated with different concentrations of treatment agents. When desired treatment duration (24, 48 or 72 h) was reached, the treatment medium was removed. Then, the cells were washed twice with PBS, and MTT solution (final concentration, 0.5 mg/mL) was added into each well. After incubation for 4 h at 37°C, the medium was slowly removed, and 300 µL DMSO was added to dissolve the formazan crystals. The percent cell viability was calculated based on the absorbance measured at 570 nm by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA), and normalized to control.

**Western blot analysis**

After treatment, the AGS cells were collected by trypsinization and lysed with cold RIPA lysis buffer. Protein quantification was conducted with a Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The lysates (20-40 µg) were subjected to 7.5-15% sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and transferred to PVDF or NC membranes using a Power Pac power supply (Bio-Rad). After blocking with 5% skim milk or 5% BSA for 1 h, the membranes were incubated with the designated primary antibodies overnight at 4°C. The membranes were washed three times with TBST (1% Tween 20) and incubated with secondary HRP-labeled antibodies in a 1:5000 dilution to detect the expression of the proteins of interest. Protein bands were examined using an ECL reagent (Santa Cruz Biotechnology) by the Bio-Rad Universal Hood II Gel doc system or X-ray film method. Image J (NIH Image, MD, USA) was used to analyze the data.

**Immunocytochemistry staining**

Immunocytochemistry for LC3B and LAMP2 was performed after the AGS cells were fixed with cold 100% methanol. After blocking with normal goat serum, the cells were incubated with the primary antibody overnight at 4°C followed by incubation with Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature. Then, the nuclei were stained with 300 nM of DAPI for 5 min at room temperature. The cells were observed and imaged using a confocal microscope (Leica) or a confocal microscope (Zeiss, Oberkochen, Germany).

**Acridine orange staining**

AGS cells were seeded with a complete medium at a density of approximately 3×10^5 cells in each confocal dish overnight. The next day, the cells were treated with different concentrations of BITC for 24 h and washed with PBS (10%FBS) once. After 20 min staining with 1 µg/mL acridine orange dye at room temperature, the fluorescence pattern was observed within 1 h under a confocal microscope (Zeiss).

**Cathepsin activity assay**

The supernatant from AGS cell lysates was incubated with omnicathepsin fluorogenic substrate (Z-FR-AMC, 100 µM) for 30 min at 37°C. The fluorescence emitted by released AMC was quantified in a plate reader (exc: 380 nm; em: 440 nm) and normalized by total protein content.

**RfNA interference**

AGS cells were transfected with either 50 nM siRNA against LC3B or control siRNA using lipofectamine RNAiMAX reagent according to the manufacturer’s instructions. The cells were then incubated with 10 µM BITC, and further analysis was conducted.

**Statistical analysis**

Data were expressed as the mean ± SEM of at least three independent experiments. Statistical differences between the groups were calculated using the Student’s t-test. Results were considered statistically significant for p values <0.05.

**RESULTS**

**BITC inhibited AGS cell viability in time and concentration-dependent manners and induced apoptotic features**

The cytotoxic effect of BITC in AGS cells was initially confirmed by an MTT assay. AGS cells were treated with 2.5, 5, 10, 20, or 40 µM BITC for 24 or 48 h. Then, the MTT assay was conducted to quantify the reduction in AGS cell viability.
As shown in Fig. 1A, BITC reduced the AGS cell viability in time and concentration-dependent manners. The IC50 measurements for 24 h and 48 h were approximately 10 µM and 5 µM, respectively. Next, the cellular morphology was observed under a fluorescent microscope, and apoptotic features such as cell shrinkage, cell rounding, and apoptotic bodies were detected after treating the cells with BITC for 6 h (Fig. 1B). The nuclear morphological changes were also observed with a nuclear dye: DAPI under a fluorescent microscope (Fig. 1C). As a result, the number of the cells with condensed nuclei was increased significantly in a concentration-dependent manner.

BITC increased the expression levels of LC3B and p62 proteins in AGS cells

To explore the effect of BITC on autophagy, the levels of the commonly-applied autophagy marker proteins LC3B and p62/SQSTM1 were detected by western blot and qPCR. Since autophagy is a dynamic process, the levels of both proteins were examined not only in concentration-based conditions but also in time-based conditions. As shown in Fig. 2A and 2B, BITC significantly increased the expression levels of both LC3B II and p62 in time and concentration-based conditions. BITC treatment also increased their mRNA levels (Fig. 2C). Treatment with 10 µM BITC increased the LC3B mRNA level by approximately three-fold, but this did not occur with 5 and 15 µM BITC. Treatment with 5 and 10 µM BITC increased the p62 mRNA level up to approximately three-fold, but this did not occur with 15 µM BITC.

Effect of BITC on other autophagy markers: Beclin1 and Atg5-Atg12 protein expression and mTOR phosphorylation in AGS cells

Since autophagy is as a multi-step mechanism, the other autophagy-related proteins involved in different steps of autophagy, such as Beclin1, Atg5-Atg12 complex, and mTOR phosphorylation were analyzed by western blotting. As shown in Fig. 3, BITC decreased the expression levels of Beclin1 and Atg5-Atg12. There was a concentration-dependent decrease in Beclin1 expression. Approximately 50% and 90% of Beclin1 expression were depleted in 10 µM and 15 µM BITC-treated groups, respectively, compared to the control. For Atg5-Atg12 complex, only a high concentration 15 µM BITC significantly decreased its expression to half, compared to control group. On the other hand, statistically significant data was not obtained for mTOR phosphorylation detection.

BITC-induced LC3B conjugation in AGS cells was not dependent on class III PI3K

The expression level of class III PI3K was measured after treating the cells with BITC for 24 h, and a potent PI3K inhibitor called wortmannin was utilized to observe the changes in LC3B conjugation level. The results showed that 15 µM BITC significantly reduced the class III PI3K protein level by approximately 50% compared to the control group (Fig. 4A). In the presence of wortmannin, the class III PI3K protein expression was significantly decreased by approximately 80%, compared to BITC monotherapy (Fig. 4A). An MTT assay was also performed to examine the cytotoxicity of wortmannin, and 24-h treatment of the typical working concentrations of wortmannin (0.2 to 1 µM) showed a 20% maximum cell death in AGS.
cells (Fig. 4B). Next, the LC3B conjugation (LC3B II level) was detected in the BITC-treated cells with or without wortmannin. As shown in Fig. 4C, wortmannin could not block the BITC-induced LC3B conjugation, and in fact, it unexpectedly enhanced the expression of LC3B II.

BITC reduced the lysosomal cathepsin activity and caused lysosomal swelling in AGS cells

The degradation step of autophagy was investigated in AGS cells under BITC treatment. The activity of the cathepsin proteins was examined in BITC-treated AGS cells to evaluate the function of lysosomes. The cathepsin activity was quantified with an omnicathepsin assay in concentration-based or time-based conditions. As shown in Fig. 5A and 5B, BITC decreased the cathepsin activity of AGS cells in a concentration-dependent manner, but not in a time-dependent manner. Bafilomycin A1 was used as a positive control to inhibit lysosomal degradation. AGS cells were treated with BITC in the presence or absence of bafilomycin A1, and the cathepsin activity was compared. As shown in Fig. 5C, bafilomycin A1 alone could reduce the basal cathepsin activity of AGS cells by approximately 60%. BITC treatment alone reduced the cathepsin activity by approximately 40% compared to the control group. In the combined treatment groups, the cathepsin activity was further decreased compared to the control group.

Fig. 2. BITC increased the expression levels of LC3B and p62 proteins in AGS cells. (A) AGS cells were treated with 10 µM BITC for 8, 12, or 24 h. Whole cell lysate was prepared, and LC3B and p62 levels were determined by western blotting. β-actin was used as a loading control. (B) AGS cells were treated with 5, 10, or 15 µM BITC for 24 h. Whole cell lysate was prepared, and LC3B and p62 levels were determined by western blotting. β-actin was used as a loading control. (C) AGS cells were treated with 5, 10, or 15 µM BITC for 12 h and mRNA levels were assessed by q-PCR. Data were calculated from three independent experiments and expressed as the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 versus the control.

Fig. 3. Effect of BITC on other autophagy markers: Beclin1 and Atg5-Atg12 protein expression and mTOR phosphorylation in AGS cells. AGS cells were treated with 5, 10, or 15 µM BITC for 24 h. Whole cell lysate was prepared, and Beclin1, Atg5-Atg12, and p-mTOR/mTOR levels were determined by western blotting. β-actin was used as a loading control. Data were calculated from three independent experiments and expressed as the mean ± SEM. ***p<0.001 versus the control.
Fig. 4. BITC-induced LC3B conjugation in AGS cells was not dependent on class III PI3K. (A) For the single treatment, AGS cells were treated with 2.5, 5, 10, or 15 μM BITC for 24 h. For the combined treatment, the cells were pre-treated with 0.5 μM wortmannin for 2 h followed by 10 μM BITC for 24 h. Then, the whole cell lysate was prepared, and the class III PI3K protein level was measured by western blotting. β-actin was used as a loading control. (B) The cytotoxicity of wortmannin was detected by MTT assay after treating the cells with different concentrations of wortmannin for 24 and 48 h. (C) AGS cells were pre-treated with 0.25 or 0.5 μM wortmannin for 2 h followed by 10 or 15 μM BITC for 24 h. After cell lysis, the change in LC3B II level was detected by western blotting. LC3B-II conjugation in AGS cells was not dependent on class III PI3K. (A) For the single treatment, AGS cells were treated with 5, 10, or 15 μM BITC for 24 h. Whole cell lysis was performed, and the supernatant was incubated with omnicathepsin (100 μM) for 30 min at 37°C. The emitted fluorescence was quantified in a plate reader (exc: 380 nm, em: 440 nm) and normalized by total protein concentration. (B) AGS cells were treated with 10 μM BITC for 6, 12, or 24 h. Whole cell lysis was performed, and the supernatant was incubated with omnicathepsin (100 μM) for 30 min at 37°C. The emitted fluorescence was quantified in a plate reader (exc: 380 nm, em: 440 nm) and normalized by total protein concentration. (C) AGS cells were treated with 5, 10, or 15 μM BITC for 24 h, stained with 1 μg/ml AO for 20 min at room temperature, and visualized by a confocal microscope within 1 h (scale-bar=50 μm). AO emits red fluorescence in the acidic compartment and green fluorescence in the cytoplasm. Data were calculated from three independent experiments and expressed as the mean ± SEM. **p<0.01 and ***p<0.001 versus the control.

Fig. 5. BITC reduced the lysosomal cathepsin activity and caused lysosomal swelling in AGS cells. (A) AGS cells were treated with 5, 10, or 15 μM BITC for 24 h. Whole cell lysis was performed, and the supernatant was incubated with omnicathepsin (100 μM) for 30 min at 37°C. The emitted fluorescence was quantified in a plate reader (exc: 380 nm, em: 440 nm) and normalized by total protein concentration. (B) AGS cells were treated with 10 μM BITC for 6, 12, or 24 h. Whole cell lysis was performed, and the supernatant was incubated with omnicathepsin (100 μM) for 30 min at 37°C. The emitted fluorescence was quantified in a plate reader (exc: 380 nm, em: 440 nm) and normalized by total protein concentration. (C) AGS cells were treated with or without 100 nM bafilomycin A1 for 24 h. Whole cell lysis was performed, and the supernatant was incubated with omnicathepsin (100 μM) for 30 min at 37°C. The emitted fluorescence was quantified in a plate reader (exc: 380 nm, em: 440 nm) and normalized by total protein concentration. (D) AGS cells were treated with 5, 10, or 15 μM BITC for 24 h, stained with 1 μg/ml AO for 20 min at room temperature, and visualized by a confocal microscope within 1 h (scale-bar=50 μm). AO emits red fluorescence in the acidic compartment and green fluorescence in the cytoplasm. Data were calculated from three independent experiments and expressed as the mean ± SEM. **p<0.01, ***p<0.001 versus the control, and #p<0.05, ###p<0.001 versus BITC treatment alone.

activity significantly declined by approximately 80% or 90% compared to the control. However, BITC did not reduce the red fluorescence or increase the green fluorescence of AO. In addition, the sizes of the red dots, which represent the acidic vesicles, were noticeably larger (Fig. 5D).
BITC increased the expression level of lysosomal membrane protein LAMP2 in AGS cells

To confirm the lysosomal swelling, the expression level of lysosomal membrane protein LAMP2 was analyzed by western blotting and immunocytochemistry staining. Under the same conditions as the previously described experiments, AGS cells were exposed to 5, 10, or 15 μM BITC for 24 h, and the expression level of LAMP2 was measured by western blotting. For immunostaining, the cells were treated with BITC for 7 h. DAPI was used as a counterstain to stain the nuclei. The western blot analysis showed that 24-h BITC treatment significantly increased the expression level of LAMP2 in a concentration-dependent manner (Fig. 6A), whereas no difference was visualized through immunostaining in the 7-h BITC-treated cells (Fig. 6B).

BITC reduced the quantity of mature cathepsins D, L, and B in AGS cells

To further confirm the lysosomal dysfunction induced by BITC, the expression levels of cathepsin proteins D, L, and B were measured by western blotting. As shown in Fig. 7A, after 24-h BITC treatment, pro-cathepsin D expression dramatically increased up to 4-fold. Prepro-cathepsin D expression seemed to increase slightly, but no statistically significant data was obtained. Mature-cathepsin D level was decreased by approximately 2%, 24%, and 28% after BITC treatments of 5, 10 and 15 μM, respectively, compared to the control. Similarly, 5 μM BITC increased the expression of pro-cathepsin L.
to almost twice that of the control group. On the other hand, 10 and 15 µM BITC treatments reduced mature-cathepsin L expression by approximately 40% compared to the control group (Fig. 7B). As shown in Fig. 7C, BITC decreased the expression level of mature-cathepsin B by approximately 10%, 20%, and 70% in 5, 10, and 15 µM BITC-treated groups, respectively, compared to the control group.

**Additive effect of BITC and bafilomycin A1 on LC3B accumulation in AGS cells**

Next, the lysosomal degradation inhibitor bafilomycin A1 was utilized, and the LC3B II accumulation pattern was assessed by western blotting and immunocytochemistry staining. As shown in Fig. 8A and 8B, the combined treatment of BITC and bafilomycin A1 showed a remarkable additive effect on LC3B II accumulation. Compared to 10 µM BITC treatment alone, LC3B II level increased by approximately 1.5 times in the presence of bafilomycin A1 in western blotting. The cytotoxicity of bafilomycin A1 was evaluated by an MTT assay, and it was confirmed that the applied concentration and duration of bafilomycin A1 treatment (100 nM, 24 h) did not induce cytotoxicity to AGS cells (Fig. 8C).

**Inhibition of different stages of autophagy and its effect on the cytotoxicity of BITC in AGS cells**

After confirming the BITC-induced autophagy inhibition through lysosomal dysfunction, the role of basal autophagy in AGS cells was determined using an MTT assay. The difference in cell viability level was detected by further inhibiting autophagy with different autophagic stage inhibitors: wortmannin to inhibit autophagy initiation, and Bafilomycin A1 to block the autophagy degradation. As shown in Fig. 9A and 9B, wortmannin significantly increased BITC-induced cell death, but bafilomycin A1 did not.

**No relationship between BITC-induced LC3 B accumulation and cytotoxicity**

Next, the probable relationship between BITC-induced LC3B accumulation and cell death was investigated. Specific siRNA was used to knock down the LC3B gene. The cells were transfected with the LC3B siRNA for 12 h, followed by an 8-h BITC treatment. Transfection efficacy was confirmed by the almost depleted LC3B protein expression observed via western blotting. A control siRNA was used as a negative control (Fig. 10A). An MTT assay was continuously conducted, and the changes in cell viability in the 24-h BITC-treated cells with and without LC3B siRNA were determined. As shown in Fig. 10B, the knockdown of LC3B did not influence the cell death induced by BITC.

**DISCUSSION**

BITC was known to have anticancer effect, and our previous study demonstrated that the mechanism of anticancer effect results from inducing apoptosis (Han et al., 2019). On the other hand, autophagy has a relationship with cancer. In this study, we focus on the tumor promoting effect of autophagy.
Furthermore, it was reported that autophagy maintains the survival of gastric cancer cells and affects the resistance of anti-cancer drugs (Cao et al., 2019). Here, we showed that BITC was related to autophagy as a new anticancer mechanism of BITC.

Autophagy has a dual role in gastric cancer as either a tumor suppressor or tumor promoter, depending on the stress conditions (Cao et al., 2019). In the relationship between autophagy and apoptosis, autophagy often occurs in a sequence that precedes apoptosis because stress often stimulates an autophagic response (Mariño et al., 2014). However, there is an also inhibitory crosstalk between autophagy and apoptosis. Autophagy can reduce a possibility to undergo apoptosis (Jung et al., 2020). Damaged mitochondria which can activate the apoptotic programmed cell death were removed by mitophagy which is selective autophagy of mitochondria (Youle and Narendra, 2011). In addition, autophagy can also attenuate apoptosis by selectively reducing the amount of pro-apoptotic proteins in the cytoplasm (Mariño et al., 2014).

In previous research, BITC has different effects for each cell type. BITC induces protective autophagy in lung cancer cells (Zhang et al., 2017), prostate cancer cells (Lin et al., 2013), and colon cancer cells (Liu et al., 2017) but BITC causes autophagic cell death in breast cancer cells (Xiao et al., 2012).

In our previous study, we found that BITC induced both intrinsic and extrinsic autophagy in gastric cancer cells. In addition to apoptosis, the present study found that BITC increased the protein expression levels of both LC3B II and p62 in time and concentration-dependent manners. LC3 and p62 are well known and commonly applied autophagy markers (Mizushima et al., 2010). When autophagy degradation occurs, LC3 II detaches from the outer membrane while both LC3 II from inner membrane and p62 are degraded along with the cargo by lysosomal proteases (Mizushima et al., 2010). Some studies reported that both LC3 and p62 proteins increased while the autophagic degradation was inhibited (Pugsley, 2017; Yi et al., 2018). In the current study, BITC induced the accumulation of both LC3B II and p62 proteins in AGS cells, suggesting that BITC may impair autophagic degradation. On the other hand, BITC increased the mRNA levels of both LC3B and p62 in AGS cells, which suggests that the host AGS cells may generate more autophagy proteins as a response against the BITC-induced stress.

Meanwhile, BITC did not alter mTOR phosphorylation, but it significantly decreased the levels of both Beclin1 and Atg5-Atg12 proteins in AGS cells. mTOR is an upstream negative regulator of autophagy, and the inhibition of mTOR phosphorylation enhances autophagy (Dossou and Basu, 2019; Wang and Zhang, 2019). On the other hand, Beclin1 is involved in the initiation step of autophagy and is essential for autophagy (Liang et al., 1999). It forms a regulatory complex with class III PI3K and recruits the required proteins for autophagosome formation (Acevo-Rodríguez et al., 2020). The Atg5-Atg12 complex functions as a ubiquitin E3-like enzyme to convert LC3 I to LC3 II, which is required for the autophagosome membrane elongation process (Green and Llambi, 2015). Accordingly, it was assumed that the lack of effect on p-mTOR and the decreased expression levels of both Beclin1 and Atg5-Atg12 complex were due to the BITC-induced inhibition of the autophagy initiation step. Consistent with the present study, other researchers have observed autophagy inhibition through the reduction of Beclin1 and Atg5 proteins (Chen et al., 2018; Rubio and Mohr, 2019).

Furthermore, the results showed that 15 µM BITC decreased the class III PI3K protein expression in AGS cells. Notably, the LC3B II accumulation induced by BITC was enhanced in the presence of wortmannin, a potent PI3K inhibitor. Evidence has shown that class III PI3K is essential for LC3 conjugation (conversion of LC3 I to LC3 II) to initiate the autophagy process (Jaber and Zong, 2013; Ge et al., 2014; Pasquier et al., 2015; Brier et al., 2019). Thus, the decrease in class III PI3K following BITC treatment suggested that BITC decreased the autophagy initiation. Moreover, since the increased LC3B II level induced by BITC was still high in the presence of a class III PI3K inhibitor, it was assumed that BITC induced LC3B conjugation did not depend on class II P3K. Consistent with the present study, another study reported that even in class III PI3K-null MEF cells, LC3 conjugation still occurred while autophagy degradation decreased (Jaber and Zong, 2013). Therefore, it was hypothesized that BITC-induced LC3 II accumulation in AGS cells may not act as an autophagy induction signal and may be a consequence of decreased autophagy degradation.

Lysosomes play a key role in autophagic degradation (Yim and Mizushima, 2020). In this study, BITC reduced the cathepsin activity of AGS cells in concentration and time-dependent manners. Moreover, a synergistic decrease in cathepsin activity was found in the combined treatment of BITC and bafilomycin A1, a specific inhibitor of V-ATPase that strongly inhibits the acidification of lysosomes and blocks lysosomal degradation (Yoshimori et al., 1991). Consistent with the results of a qualitative assay, a western blot analysis showed a significant
In this study, the relative toxicity of BITC in normal cells was not examined due to limited time and resources. However, toxicity testing of BITC in normal cells has already been conducted in several studies. In one example, BITC inhibited cell growth, promoted G2/M phase arrest, and triggered apoptosis of oral cancer OC2 cells, with a minimal toxicity to normal PBMCs (Yeh et al., 2016). Similarly, BITC has been found to induce apoptosis in breast cancer cells but has no effect on normal breast MCF-10A cells (Sehrawat et al., 2016). These studies suggested that BITC has selective toxicity to tumor cells and is safe to be used for cancer treatment.

A previous study reported on the novel role of LC3-induced non-autophagic cell death in 15d-PGJ2 treated cancer cells (Kar et al., 2009). Thus, the present study examined the difference in cell viability in the presence and absence of BITC-induced LC3B accumulation by knocking down the LC3B gene with siRNA. No significant difference was found in the cell viability in the presence or absence of LC3B siRNA, suggesting an indirect relationship between the BITC-induced LC3B upregulation and the cytotoxicity in AGS cells.

In summary, the BITC-induced AGS cell death mechanism involves autophagy inhibition in addition to its apoptotic effect. BITC could inhibit the cytoprotective autophagy of AGS cells by decreasing both the autophagy initiator proteins and the lysosomal degradation. The results of this study provide a basic for BITC as a novel anti-cancer agent and support autophagy as a promising target for gastric cancer treatment (Fig. 11).

**CONFLICT OF INTEREST**

There is no conflict of interest to declare.

**ACKNOWLEDGMENTS**

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology [Grant NRF-2019R1F1A1062070], the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.NRF-2021R1F1A1050004), and the Chung-Ang University Young Scientist Scholarship (CAYSS) program in 2017.
REFERENCES


Po et al. Effect of BITC on Autophagy in AGS Cells


