Involvement of phosphatidylinositide 3-kinase pathway in the resistant mechanisms against benzyl isothiocyanate in human colorectal cancer cells

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PREFACE

The experiments described in this dissertation were carried out at the Graduate school of Environment and Life Science (Doctor Course), Okayama University, Japan, from October, 2014 to September 2017, under the supervision of Professor Y. Nakamura. These studies are original work by the author and any other assistance and collaboration from others are specially acknowledged.

This dissertation has not been submitted previously whole or in part to the council, a university or any other professional institution for a degree, diploma or other professional qualification.

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ABBREVIATIONS

PI3K, phosphatidylinositide 3-kinase;

Akt/PKB, protein kinase B;

FoxO1, forkhead box protein O1;

mTOR, mammalian target of rapamycin;

PIK3CA, PI3K catalytic subunit, alpha isoform;

ITCs, isothiocyanates;

BITC, benzyl isothiocyanate;

MAPK, mitogen-activated protein kinases;

DMEM, Dulbecco's modified Eagle's medium;

IRβ, insulin receptor β;

PTP1B, protein-tyrosine phosphatase 1B;

PI, propidium iodide;

ERK, extracellular signal-regulated kinase;

MEK, MAPK/ERK kinase;

Nrf2, Nuclear factor-erythroid 2 (NF-E2)-related factor 2 ;

Keap1, kelch-like ECH-associated protein 1;

ARE, antioxidant response element;

SQSTM1, sequestosome 1

LC3, microtubule-associated protein 1 light chain 3

ABSTRACT

Phosphatidylinositide (PI) 3-kinase, a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit, possesses activities that have been subsequently found in eukaryotic cell types and are involved to an incredible diverse characteristic of key cellular functions, including cell growth, proliferation, motility, differentiation and survival. An emerging association between PI3K and human diseases, especially cancer, brings more and more attention of focus on intense study. An increasing number of human cancer cell lines has been proved to harbor PI3K mutant, resulting in easily occurring shift of anti-drug cellular characteristic caused by simulative activation and overexpression of PI3K. Thus, inhibition of PI3K is considered potential therapeutic options. Food phytochemicals, such as organosulfur compound, have considered as promising candidates for health promoting food chemicals in recent years. Among them, benzyl isothiocyanate (BITC), one of the isothiocyanate (ITC), has been shown to have antiproliferative properties against a variety of cell lines. Based on this concept, to investigate BITC as an anti-cancer agent against PI3K-related cellular responses, I demonstrate that regulation of PI3K is an effective strategy for controlling PI3K-associated cellular pathway and activities.

In Chapter 2, I clarified the role of phosphoinositide 3-kinase (PI3K) in antiproliferation induced by benzyl isothiocyanate (BITC) in human colorectal cancer cells (HCT-116, HT-29, DLD-1). BITC simultaneously activated the PI3K/Akt/forkhead box O (FoxO) pathway, whereas it significantly inhibited the proliferation in HCT-116 cells. Inhibitory experiments using a PI3K selective inhibitor, LY294002 or NVP-BEZ235, significantly enhanced the BITC-induced antiproliferation and apoptotic cell population with the attenuation of the BITC-induced activation of the PI3K/Akt/FoxO survival pathway. Furthermore, BITC enhanced the insulin-activated PI3K/Akt/FoxO pathway, possibly through its inhibition of the protein tyrosine phosphatase 1B enzymatic activity. Taken together, these results suggested that the

PI3K/Akt/FoxO pathway negatively regulates the BITC-induced antiproliferation in human colorectal cancer cells.

In chapter 3, I investigated the regulating role of autophagy in benzyl isothiocyanate (BITC)-induced Nrf2 activation, laying a pivotal role in the inducible expression of cytoprotective genes. BITC not only Time-dependently (12h) but also dose-dependently induced autophagy, concomitantly with Keap1/Nrf2 modulation in human colorectal cancer HCT-116 cells. Immunofluorescence results suggested that BITC induced the formation of autophogosome and lysosome, which is also confirmed by positive BITC-induced formation of autophagic vacuoles by using MDC staining. Experiments using a phosphatidylinositide 3-kinase (PI3K)-specific inhibitor suggested inhibition of PI3K not only attenuated BITC-induced accumulation of autophagic molecules, but also compromised autophagy-related p62-Nrf2-Keap1axis induced by BITC. On the other hand, inhibitory experiments revealed that HO-1 gene, a cytoprotective gene, was also involved in PI3K-related autophagic induction. Taken together, these results suggested that PI3K plays the key role in the autophagy-dependent Nrf2 activation by BITC.

My findings provide biological evidences that (1) regulation of PI3K plays an essential role not only in the BITC-induced PI3K/Akt survival pathway but also in the enhancement of anti-proliferative effects by BITC against BITC-induced drug-resistance in HCT-116, HT-29 and DLD-1 human colon cancer cells; (2) PI3K is involved in induction of autophagy by BITC and also regulates autophagy-related p62/Nrf2/Keap1 axis and expression of cytoprotection genes in HCT-116 human colon cancer cells. Taken together, these studies proposed that regulation of PI3K is a promising strategy to overcome resistance of food-derived compounds activating PI3K/Akt and Nrf2 pathway in human colon cancer cells.

CHAPTER 1

General Introduction

1.1 Benzyl isothiocyanate

A number of researches support that certain food phytochemicals protect against cancer. An important group of chemicals that possess this property are organosulfur compound, such as isothiocyanates (Fahey et al., 2001). Isothiocyanates, naturally occurring in abundance in cruciferous vegetables such broccoli, watercress, Brussels sprouts, cabbage, Japanese radish, and cauliflower, may plays a significant role in affording the cancer chemopreventive properties of these vegetables (Nakamura et al., 2007b). Based on these anti-cancer properties that ITCs have, through different mechanism including induction of phase 2 detoxifying enzyme, induction of apoptosis, inhibition of cell cycle progress and induction of anti-inflammatory activity (Miyoshi et al., 2004a; Nakamura et al., 2002), ITCs exhibit a promising cancer chemotherapeutic effects on a variety of cancer cell types. Benzyl isothiocyanate (BITC), an isothiocyanate compound which is a hydrolysis product of the glucosinolate glucotropaeolin (Bennett et al., 1997) derived from cruciferous vegetables, has been shown to have anti-carcinogenic properties. BITC is also potent in suppressing proliferation by causing DNA damage, G2/M cell arrest and apoptosis in many cancer cell lines, including pancreatic cancer (Sahu et al., 2009) and prostate cancer (Lin et al., 2013).

1.2 Colorectal cancer

Colorectal cancer (CRC), also known as bowel cancer or colon cancer, has been announced to be the second most common cancer worldwide by the World Health Organization and CDC. Due to its mortality rate, CRC persists as one of the most deadly and prevalent tumor types in both women and men around the world (Hammond et al., 2016). Including CRC, metastatic disease is considered incurable. In spite of advances in systemic therapy, the 5-year survival rate is still a mere 12.5% (Siegel et al., 2014), even though patients are typically given a combination of cytotoxic chemotherapy with a target

therapy. The primary reason for cancer failure are commonly considered to be an acquired resistance that contributes to nearly 90% of the patients with such occurring anti-drug resistance during the chemotherapeutic treatment (Longley and Johnston, 2005).

1.3 Drug resistance

Malignant tumor can have intrinsic resistance and/or acquired resistance and each of them is essential in determining initial and subsequent lines of treatment. Between these two types of resistance, tumors might intrinsically initialize drug-resistance or develop acquired resistance to chemotherapy during treatments (Longley and Johnston, 2005). Acquired resistance is a particular problems to patients, as tumors not only turn into resistant to original treatment, but also can obtain cross-resistant to

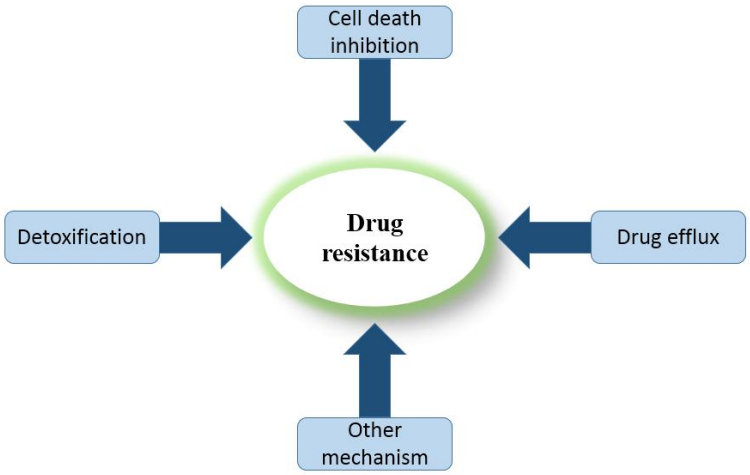


Fig. 1.1 Schematic model of drug-induced cell resistance

the other chemotherapy with different mechanism of actions. These factors are believed to contribute to a fact that the drug-treatment failure remains still stubbornly high with even multiple kinds of mechanism of chemotherapy during the treatments.

In the chemotherapeutic process, each cytotoxic therapy and each targeted pathway may result in different mechanism of acquired resistance, but acquired resistance to one

drug often confers resistance to the other drug that even acts in a different targeting mechanism, which refers to a concept as multidrug resistance (MDR) (Hammond et al., 2016). Different mechanism is involved in resistance to targeted therapies, including upregulation, mutation or activation of downstream signaling molecules within specific pathways (Tejpar et al., 2012). The shortage of understanding the mechanisms of acquired drug resistance to targeted therapies still remains to issues that obstructively develop future therapies.

1.4 PI3K/Akt survival pathway

Phosphatidylinositide 3-kinase (PI3K)/Akt is a potential survival pathway that may regulate resistance to the apoptotic effects of chemotherapy drugs and radiation therapy in a variety of cancer cell lines (Boreddy et al., 2011). Despite how much advancement in chemo- and radio- therapy, PI3K/Akt pathway still remains highly reality of anti-drug properties to clinical treatment. Such existence of PI3K/Akt-related resistance could be contributed by hyperactivation of Akt in particular cell lines, or ether be activated exogenously by growth factor, cytokines or anti-cancer reagent (Hossini et al., 2016). Both apoptosis and survivals of cells could be controlled by proteins including Akt involved in PI3K pathway. After full activation of Akt, phosphorylated Akt enhances survival and inhibits apoptosis through phosphorylation and deactivation of several anti- and pro- apoptotic target proteins, including mTOR, FoxO families, pro-apoptotic BAD, a regulative ratio of Bcl-2 and BAX genes and tumor suppressor p53.

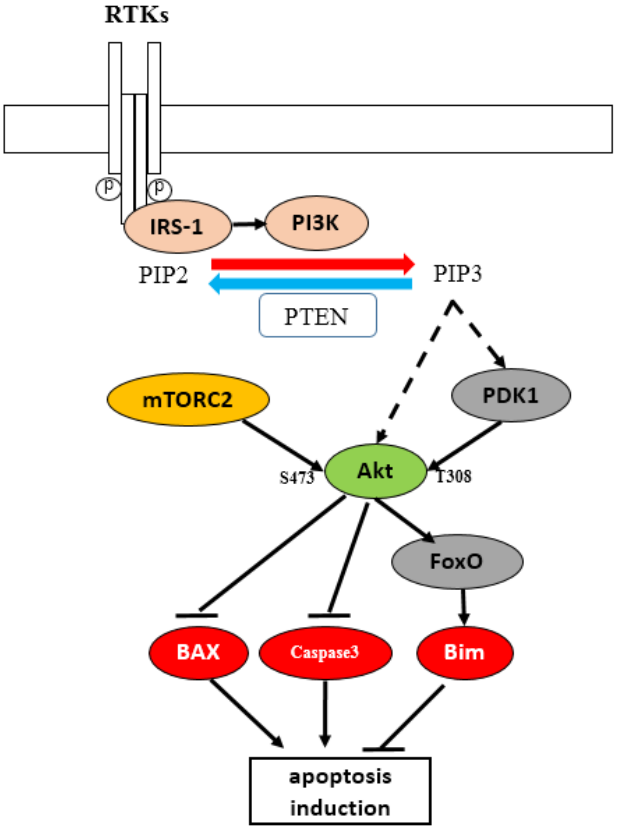


Fig. 1.2 Schematic model of PI3K/Akt/FoxO survival pathway

1.4.1 Receptor tyrosine kinase

Receptor tyrosine kinases (RTKs), a family of cell surface receptors, have emerged as key regulators of critical cellular processes, such as proliferation and differentiation, cell survival and metabolism, cell migration, and cell cycle control (Blume-Jensen and Hunter, 2001). All RTKs have a similar molecular architecture, with a ligand-binding region in the extracellular domain, a single transmembrane helix, and a cytoplasmic region that contains the protein tyrosine kinase (TK) domain plus additional carboxy (C-) terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger, 2010). Numerous human diseases, including cancers, diabetes, inflammation, have been causally linked to mutation of RTKs and aberrant activation of its intracellular signaling pathway that alter cellular activities. Among these RTKs, the insulin receptor and IGF-1 are expressed on the cell surface as disulfide-linked $(\alpha\beta)_2$ dimers (Ward et al., 2007). Binding of insulin and IGF-1 results in structural changes and stimulates tyrosine kinase activity,

which leads to activation of the intracellular tyrosine kinase domains and cell signaling pathway, including PI3K/Akt survival pathway.

1.4.2 Role of Akt in PI3K/Akt pathway

Akt/protein kinase B (PKB) is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation, which has emerged as central role in the signaling transduction (Brazil and Hemmings, 2001). Two phosphorylation sites, Th308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domains, are contained in all the Akt/PKB isoforms except for Akt3. Activation of PI3-kinase by stimuli, such as growth factor and anti-cancer reagent, contributes to activation of Akt at the Thr308 site through signaling transduction of PDK1. Phosphorylation of Thr308 partially activates Akt, while full activation needs phosphorylation of both sites.

1.4.3 Transcriptional factor FoxO

The Forkhead (FoxO) family of transcriptional was first identified from genetic analysis of C.elegans (Paradis and Ruvkun, 1998), which has now been identified in several different organism, including Caenorhabditis elegans, zebrafish, Drosophila, mouse, rat and humans (Van Der Heide et al., 2004). FoxO factors can regulated by several signal transduction cascades. Among these cascades, PI3K pathway plays a primary role in controlling the phosphorylation of FoxO factors and its intracellular localization and transcriptional functions. Once PI3K/Akt pathway is activated, the activated Akt detaches from the cellular membrane and translocates to the cytosol and nucleus, where it phosphorylates serine or threonine residues within its motif and gets ready to transduce signals to its downstream target proteins (Lawlor and Alessi, 2001). Within the structure of FoxO families, including murine FoxO1, FoxO3, FoxO4, and FoxO6, three highly conserved putative Akt recognition motifs are contained for those phosphorylation by Akt. All the FoxO proteins require the consensus N-terminal Akt site and Akt site located in the forkhead domain to translocate form nucleus to cytosol

(Brownawell et al., 2001). The phosphorylated FoxO factors can regulate cell survival thought controlling its target genes that may be important in the inhibitory effect of cell survival.

1.4.4 PTP1B and its involvement in PI3K/Akt pathway

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed phosphatase that has emerged as a relevant regulator of signaling cascade initiated by the activation of the tyrosine kinase receptor families (Haj et al., 2003). It has an N-terminal catalytic domains, two proline-rich sequences, and a C-terminal hydrophobic region (He et al., 2014). Due to its capability to dephosphorylate and inactivate the insulin receptor (IR), PTP1B is a critical role of insulin signaling through switching off insulin signaling (Salmeen et al., 2000). Recent studies have shown that small molecule inhibitors of PTP1B increase insulin-stimulated IR and IRS (insulin receptor substrate) phosphorylation (Ahmad et al., 1995; Zhang and Zhang, 2007), suggesting that inhibition of PTP1B might sensitize insulin-related pathway. In addition, given its function in dephosphorylation of RTKs that are responsible for inducing oncogenic signaling and drug-induced resistance, PTP1B has always been considered as a potential tumor suppressor. However, studies also revealed that PTP1B can promote tumorigenesis (Lessard et al., 2010). Therefore, understanding the connection between PTP1B activities and anti-cancer reagent seems to be an important potential strategy for the cancer therapy.

1.5 MEK/ERK cascade

The Ras/Raf/MEK/ERK cascade transduces signals from cell surface receptors to transcriptional factors, which regulate gene expression. Furthermore, this cascade also is responsible for regulating the activity of many proteins involved in drug-resistance and apoptosis. Due to such functions of MEK/ERK pathway, it is also considered to have profound effects on the regulation of apoptosis by the post-phosphorylation of apoptotic regulatory molecules, including Bad, Bim, caspase-9 and Bcl-2 (Mccubrey et al., 2007).

Therefore, this pathway has diverse effects that can regulate apoptosis, cell growth, cell cycle progression and differentiation. For the reasons described above, MEK/ERK has been believed to be an important pathway to target for the cancer therapy in the clinical trials. However, many studies show a dual effect on cell proliferation (Marshall, 1999). It is shown to promote cell proliferation by inducing the expression of cyclin D and enhance the activation of cell cycle kinases (Cheng et al., 1998; Lavoie et al., 1996). In addition, ERK can interfere with apoptosis on several levels, including preventing activation of caspases (Tran et al., 2001) and inducing the expression of anti-apoptotic factors. Furthermore, many anti-drugs show that they can activate MEK/ERK cascade, which has negative role in regulating cell cycle progression and drug-induced apoptotic effects (Boldt et al., 2002). Due to the discoveries above, the modulative functions of MEK/ERK to efficacy of chemotherapeutic drugs seem to be considered cell line-dependent and anti-cancer drug-dependent.

1.6 Autophagy

Autophagy is a self-degradative progress that is essential for cellular homeostasis at critical time in development and in response to nutrient condition, oxidative stress and cytotoxicity(Glick et al., 2010) through clearing damaged organelles, degrading misfolded proteins, as well as eliminating intracellular pathogens. There are three commonly defined types of autophagy: micro-autophagy, macro-autophagy and chaperone- mediated autophagy, which are considered to be responsible for different type of cellular stress, respectively. Macroautophagy delivers cytoplasmic cargo to the lysosome against streess acquired by anti-cancer chemicals through intermediation by a double-membrane vesicle, refered to as an autophagosome, which can fuse with lysosome to form an autophogolysosome that finally be able to degreade and eliminate unnecessary intracellular components. For the procedure described above, several primary steps are necessary, such as formation of autophagosome and lysosome, as well as fusion of autophagosome and lysosome (Glick et al., 2010).

1.6.1 Autophagosome and lysosome

Autophagosome is a double-membrane spherical structure that is the key structure in macroautophagy. The initial step for formation of autophagosome needs endoplasmic reticulum, followed by elongation of structures called phagophores (Kruppa et al., 2016). The formation of autophagosome is controlled by Atg genes through conjugation of Atg12-Atg5 and LC3 (Microtubule-associated protein 1A/1B-light chain 3). LC3 proteins are originally identified as one of three light chains (LC1, LC2 and LC3). During the progress of formation of autophagosomal membrane, cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) (Sou et al., 2006) by two consecutive ubiquitylation-like reaction catalyzed by the E1-like enzyme Atg7 and E2-like enzyme Atg3 (Tanida et al., 2004, 2001) to LC3- II.

Lysosomes are intracellular membrane-bound organelles that receive misfolded proteins, damaged organelles and pathogens delivered by endocytosis, phagocytosis and autophagy for degradation and recycling to maintain intracellular homeostasis (Piao and Amaravadi, 2016). Although recent studies investigated by many research groups show that luminal lysosomal hydrolases, lysosomal membrane proteins have been found to have indispensable functions. Among these proteins, more than 25 proteins have been identified (Saftig and Klumperman, 2009), with the most abundant lysosomal membrane proteins, lysosome-associated membrane protein 1 (LAMP-1) and LAMP-2, which represent 50% of all the membrane proteins within the lysosome (Saftig et al., 2010). These proteins are essential for lysosomal biosynthesis, lysosomal acidification and fusion with autophagosome. Once maturation of autophagosome and lysosome occurs, lysosome serve as the terminal station with the most acidic pH of 4.5 for the lysosome-related autophagic pathway.

1.6.2 Involvement of PI3K in autophagy

In mammalian cells, there are different classes of PI3K (phosphatidylinositide 3-kinase) that regulate autophagy. VPS34, a catalytic subunit of phosphatidylinositide 3-

kinase complexes (Itakura et al., 2008), phosphorylates phosphatidylinotisol to form phosphatidylinotisol-3-phosphate (PtdIns3P), which enables the synthesis of autophagosomes. Vps34 has been found to play an essential role in endocytosis, autophagy and mTOR activation (Burman and Ktistakis, 2010), which has been established largely by the use of the pharmacological inhibitors wortmannin and 3-methyladenine (3-MA) that are utilized as a suppressor to autophagy in many studies. However, due to these specificities of these inhibitors, the precise mechanism between Vps34 and anti-cancer reagent stills remains unclear.

1.6.3 p62-dependent Nrf2/Keap1 noncononical pathway

The nuclear factor erytheroid-derived-2-like 2 (Nrf2)-Kelch-like like ECH-associated protein 1 (Keap1) pathway is a redox and xenobiotics sensitive signaling axis that protects cells against cellular toxicity, oxidative stress and harmful chemicals through the induction of cytoprotective detoxifying genes (Jiang et al., 2015). The crosslink between the Nrf2-Keap1-antioxidant-responsive elements (ARE) axis and autophagy was reported to have association with p62/sequestosome 1 (SQSTM1) and Keap1 several times (Komatsu et al., 2010a; Lau et al., 2010). However, in 2010, it is particularly discovered that p62 activates Nrf2 by a no-canical pathway (Lau et al., 2010). p62 regulates cellular redox hemostasis by binding to Keap1 and sequester Keap1 into the autophagosomes for degradation in an autophagy-dependent manner (Komatsu et al., 2010b), which attenuates the ubiquitylation of Nrf2, leading to its free accumulation and translocation into nuclear (noncanonical pathway) (Jiang et al., 2015). Hence, Nrf2 binds to ARE in the promoter regions of detoxifying and anti-oxidant genes (Ma, 2013), such as NQO-1 and HO-1.

1.7 study outline

In the present study, I examined the effect of BITC on the phosphatidylinositide 3-kinase (PI3K)/Akt/forkhead box O (FoxO) survival pathway in human colorectal cancer cells (HCT-116, HT-29, DLD-1). I also investigated the

regulating role of autophagy in benzyl isothiocyanate (BITC)-induced Nrf2 activation, playing a pivotal role in the inducible expression of cytoprotective genes in human colorectal cancer HCT-116 cells and clarified the mediating role of PI3K in the autophagy-dependent Nrf2 activation by BITC.

My findings provide biological evidences that (1) BITC activates the PI3K/Akt/FoxO survival pathway, even though it significantly inhibits the proliferation in HCT-116 cells; (2) Inhibitory experiments using a PI3K selective inhibitor, LY294002 or NVP-BEZ235, significantly enhances the BITC-induced antiproliferation and apoptotic cell population with the attenuation of the activation of the PI3K/Akt/FoxO pathway; (3) BITC time- and dose-dependently induces autophagy, concomitantly with Nrf2 up-regulation in human colorectal cancer HCT-116 cells; (4) PI3K plays the key role in the autophagy-dependent Nrf2 activation by BITC in HCT-116 human colon cancer cells. Taken together, these studies propose that combinatory treatment with the PI3K inhibitors is a promising strategy to overcome resistance of food phytochemicals as well as anti-cancer agents, activating PI3K in human colon cancer cells.

CHAPTER 2

Inhibition of phosphatidylinositide 3-kinase ameliorates antiproliferation by benzyl isothiocyanate in human colon cancer cells

2.1 Introduction

Phosphatidylinositide 3-kinase (PI3K), a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit, catalyzes the phosphorylation of a plasma membrane lipid phosphatidylinositide-4,5-bisphosphate into phosphatidylinositide-3,4,5-trisphosphates. The phosphatidylinositide-4,5-bisphosphate phosphorylation by PI3K leads to the translocation and phosphorylation of Akt (Plastaras et al., 2008), a critical downstream target of PI3K which activates a variety of downstream targets including forkhead box O (FoxO) 1, mammalian target of rapamycin (mTOR), and ribosomal protein S6 (Schmidt et al., 2002). Full activation of Akt is achieved after phosphorylation at the active site residues of Thr^{308} and Ser^{473} by phosphoinositide-dependent kinase-1 and $mTORC_2$, respectively (Comb et al., 2012). Akt plays an important role in controlling cell survival, cell growth and cell proliferation (Zhang et al., 2016), possibly through modulating the function of numerous substrates, including $mTORC_1$ (Troca-Marín et al., 2014), nuclear factor κB, and FoxO (Coomans de Brachène et al., 2014). Among them, FoxO modulates the cell cycle arrest (induction of $p21^{Cip1}$ and $p27^{Kip1}$), apoptosis and DNA damage repair.

Drug resistance, generally categorized as intrinsic or acquired, often limits the efficacy as well as outcome of chemotherapy. In addition to the increasing efflux of the drug through ATP-dependent transporters (Gottesman et al., 2002), PI3K is another plausible molecule that mediates resistance to the chemotherapy drugs. The PI3K-mediated pathway is frequently activated (Boreddy et al., 2011; Fahy et al., 2003) and influences cell growth, survival and drug resistance (Jian et al., 2015) in a variety of

human cancer cells including colorectal cancer cells. Excessive PI3K activation is mainly caused by genetic aberrations, such as the receptor-type tyrosine kinase overexpression, PIK3CA (PI3K catalytic subunit, alpha isoform) mutations and PTEN (Phosphatase and Tensin homolog deleted from chromosome 10) mutations and deletions, which are found in about 40% of large bowel tumors (Danielsen et al., 2015). Inhibition of PI3K with a classical inhibitor, wortmannin, reduced cell growth in a soft agar assay (Khaleghpour et al., 2004) and PI3K p85 knockdown increased the sensitivity to 5-fluorouracil in colon cancer cells (Sun et al., 2009). Thus, the PI3K/Akt/FoxO pathway is one of the most attractive therapeutic targets to improve the therapeutic efficacy of anti-cancer drugs.

Organosulfur compounds including isothiocyanates (ITCs) are an important and promising group of compounds that have a cancer-preventive property (Nakamura, 2009). Benzyl isothiocyanate (BITC), an ITC compound mainly derived from cruciferous vegetables, has been shown to have antiproliferative properties against a variety of cell lines including hepatocytes (Sugie et al., 1993), T lymphocytes (Miyoshi et al., 2004b), colon fibroblasts (Miyoshi et al., 2007), cervical epithelial cells(Miyoshi et al., 2008), renal proximal tubular cells (Abe et al., 2012), and colorectal cancer cells (Abe et al., 2014; Sakai et al., 2012). We previously found that BITC inhibits cell proliferation by inducing cell cycle arrest and apoptosis mainly via the mitogen-activated protein kinase (MAPK) pathways in several human cancer cell lines (Miyoshi et al., 2004b). However, the regulating role of the PI3K/Akt/FoxO pathway in antiproliferation by BITC in human colorectal cancer cells remains unclear.

In the present study, we clarified the role of the PI3K/Akt/FoxO pathway in antiproliferation induced by BITC in human colorectal cancer cells. We demonstrated that BITC inhibited the proliferation in human colorectal cancer HCT-116 cells, whereas it activated the PI3K/Akt/FoxO pathway. Inhibitory experiments using a

PI3K selective inhibitor, LY294002 or NVP-BEZ235, indicated that inhibition of PI3K/Akt/FoxO ameliorates apoptotic cell death induced by BITC. These results suggest that the PI3K/Akt/FoxO pathway negatively regulates the BITC-induced antiproliferation in human colorectal cancer cells. The present results provide evidence that the combination of BITC with inhibitors of the survival pathway is a promising therapeutic strategy to overcome resistance.

2. 2Materials and methods

2.2.1 Cell culture

HCT-116 cells and HT-29were obtained from the American Type Culture Collection (Manassas, VA, USA). DLD-1 cells were obtained from Tohoku University Cell Resource Center for Biomedical Research (Miyagi, Japan). HCT-116, HT-29 and DLD-1 cells were maintained in DMEM (Dulbecco's modified Eagle's medium, high glucose). All medium were supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown at 37^{o}C in an atmosphere of 95 % O_2 and 5 % CO_2.

2.2.2 Chemicals and antibodies

BITC were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Antibodies against phosphorylated-insulin receptor β (IRβ) (Y1150/Y1151), phosphorylated-PI3K (Y458), phosphorylated-Akt (S473), phosphorylated-Akt (T308) and Akt were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against IRβ, PI3K, phosphorylated-Foxo (S256), Foxo, actin and horseradish peroxidase-linked anti-rabbit and anti-mouse IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LY294002 and a protein-tyrosine phosphatase 1B (PTP1B) inhibitor (CAS 765317-72-4, PTP1B inhibitor XXII) were purchased from Calbiochem (Darmstadt, Germany). NVP-BEZ235 was purchased from Selleckchem (Houston, Texas, USA). Annexin-V-FLUOS stain kit was purchased from Roche. (Mannheim, Germany). Propidium iodide (PI) and protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue stain was purchased from Life technologies (Carlsbad, CA, USA). Fatal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chemi-Lumi One Super was purchased from Nakalai Tesque Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2.3 Trypan blue dye exclusion assay

Trypan blue dye exclusion assay was carried out for quantitative analysis of cell viability. Cell suspensions were mixed with 0.4% Trypan blue stain. The total cells and viable cells (cells that excluded blue dye) were counted using a hemocytometer (Bürker-Türk, Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) under a light microscope.

2.2.4 Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline without calcium and magnesium (PBS (-)). Whole-cell lysates were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM NaH_2PO_4, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1% SDS, 1% Sodiumdeoxycholate and 1% Triton-X-100) containing protease inhibitor cocktail and left on ice for 20 min. After sonication, lysates were centrifuged and the supernatant was used as whole-cell lysates. Protein concentration in the supernatant was determined by the Bio-Rad protein assay. Equal quantities of protein were subjected to SDS-PAGE and transferred to Immobilon-P membrane. The membranes were blocked and then incubated with the primary antibody overnight at 4^oC followed by an appropriate secondary antibody. Secondary antibody binding was visualized using a Chemi-Lumi One Super. Densitometric analysis of the bands was carried out using the Image J Software Program (National Institutes of Health, Bethesda, MD, USA).

2.2.5 Cell apoptosis analysis

Cells were washed with ice-cold phosphate-buffered saline without calcium and magnesium (PBS (-)) as describe above. After centrifuge, cells were well suspended in Annexin-V-FLUOS stain kit solution and incubated in the dark at room temperature for 15 min as described in the kit manufacture. The stained HCT-116 cells were analyzed by a Tali™ image-based cytometer (Life Technologies).

2.2.6 Cell cycle analysis

Cells were washed with ice-cold phosphate-buffered saline without calcium and magnesium (PBS (-)) as describe above. After centrifuge, cells were suspended in PI solution (0.1% Triton X-100, 0.2 mg/ml RNase A, 20 μg/ml PI in Dulbecco's PBS).

2.2.7 In vitro enzymatic activity assay

Human recombinant PTP1B was incubated with the increasing concentrations of BITC and glutathione (1 mM) in the dark at 37^{o}C for 30 min. Reaction solution (37.5 mM HEPES (pH 7.5), 150 mM NaCl, 2,5 mM EDTA, 0.5 mM DTT, 0.1 % BSA, 50mM p-Nitrophenyl Phosphate) was added and then incubated for 30 min at 37^{o}C in the dark. After adding NaOH (1 N) to stop the reaction, absorbance was determined by a microplate spectrophotometer (Bio-Rad).

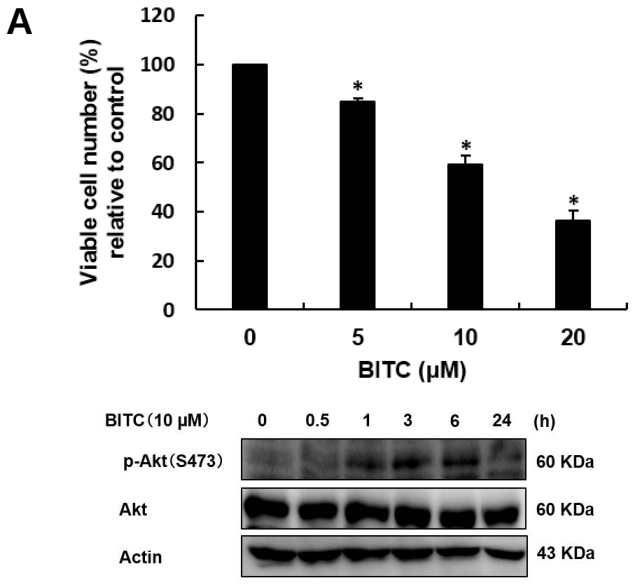
2.2.8 Statistical analysis

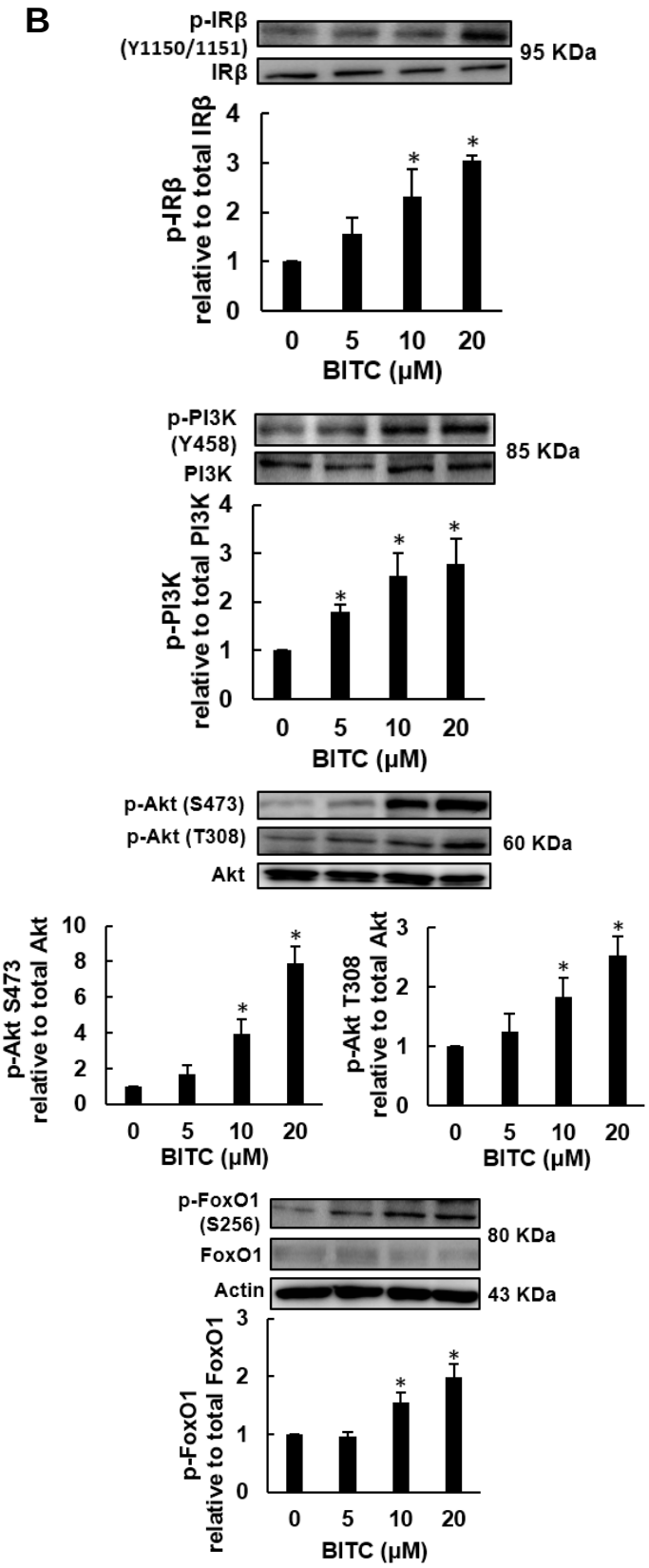
The data are expressed as the means ± standard deviation (S.D.) of at least three independent experiments and were analyzed using Student's t-test or Tukey test for comparison between groups. *P* values of < 0.05 were considered to be statistically significant.

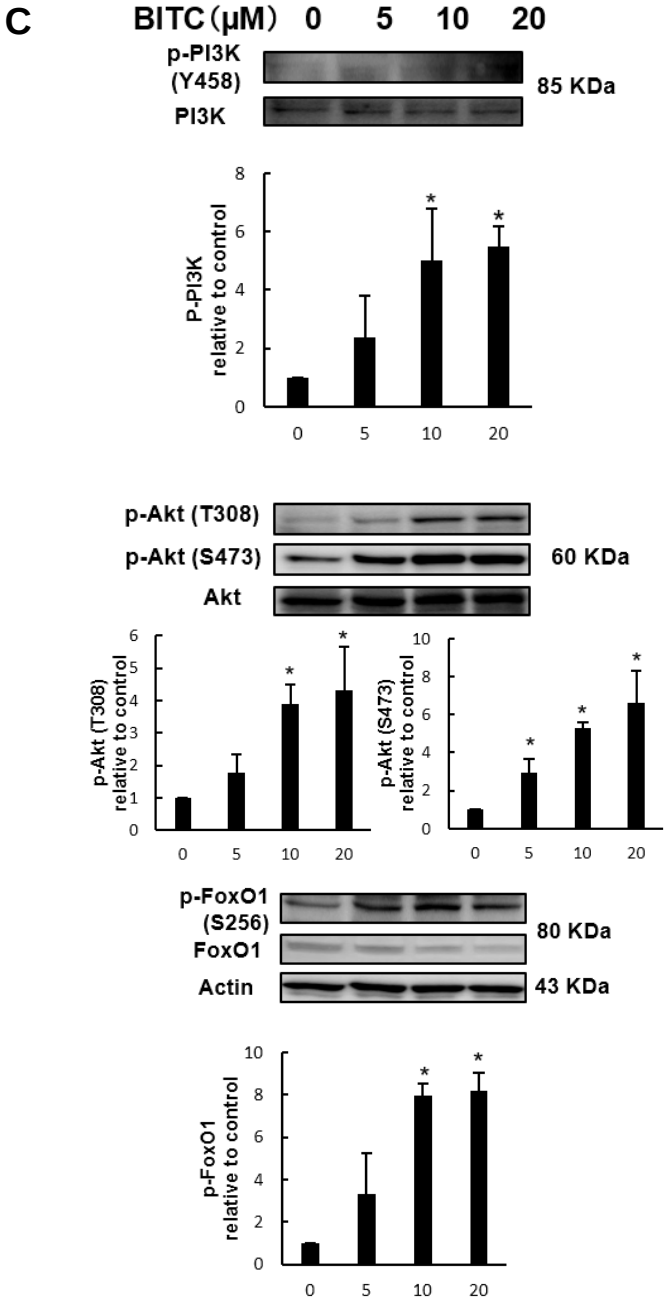
2.3 Results

2.3.1 BITC as an activator of the PI3K/Akt/FoxO survival pathway in human colon cancer cells

The HCT-116 cell line is commonly used as a colorectal cancer model because it has loss-of-function mutations in PI3KCA (Mueller et al., 2012). As previously reported (Sakai et al., 2012), BITC dose-dependently suppressed the viability of HCT-116 cells (Fig. 2.1A). Western blot analysis unexpectedly showed that the treatment of 10 μM BITC for 1 h increased the Akt phosphorylation at Ser^{473}, whereas its 24-h incubation did not affect it (Fig. 2.1A). To confirm this phenomenon, the phosphorylated protein levels of dominant molecules in the PI3K/Akt/FoxO pathway, including IRβ, PI3K, Akt and FoxO1, was examined. The 1-h incubation of BITC significantly and dose-dependently enhanced the levels of each phosphorylated protein (Fig. 2.1B&C&D). The treatment with BITC potentiated the Akt phosphorylation not only at Thr^{308}, but also at Ser^{473} as well as its downstream target, p-FoxO1, (Fig. 2.1B&C&D), suggesting that, even though BITC inhibits the proliferation in human colorectal cancer cells, it actually activates the PI3K/Akt/FoxO pathway.




B
p-IRβ
(Y1150/1151)
IRβ
95 KDa
p-IRβ
relative to total IRβ
0
1
2
3
4
0
5
10
20
BITC (μM)
p-PI3K
(Y458)
PI3K
85 KDa
p-PI3K
relative to total PI3K
BITC (μM)
p-Akt (S473)
p-Akt (T308)
Akt
60 KDa
p-Akt S473
relative to total Akt
p-Akt T308
relative to total Akt
BITC (μM)
p-FoxO1
(S256)
FoxO1
Actin
80 KDa
43 KDa
p-FoxO1
relative to total FoxO1
BITC (μM)


C
BITC（μM） 0 5 10 20
p-PI3K
(Y458)
PI3K
85 KDa
P-PI3K
relative to control
p-Akt (T308)
p-Akt (S473)
Akt
60 KDa
p-Akt (T308)
relative to control
p-Akt (S473)
relative to control
p-FoxO1
(S256)
FoxO1
Actin
80 KDa
43 KDa
p-FoxO1
relative to control

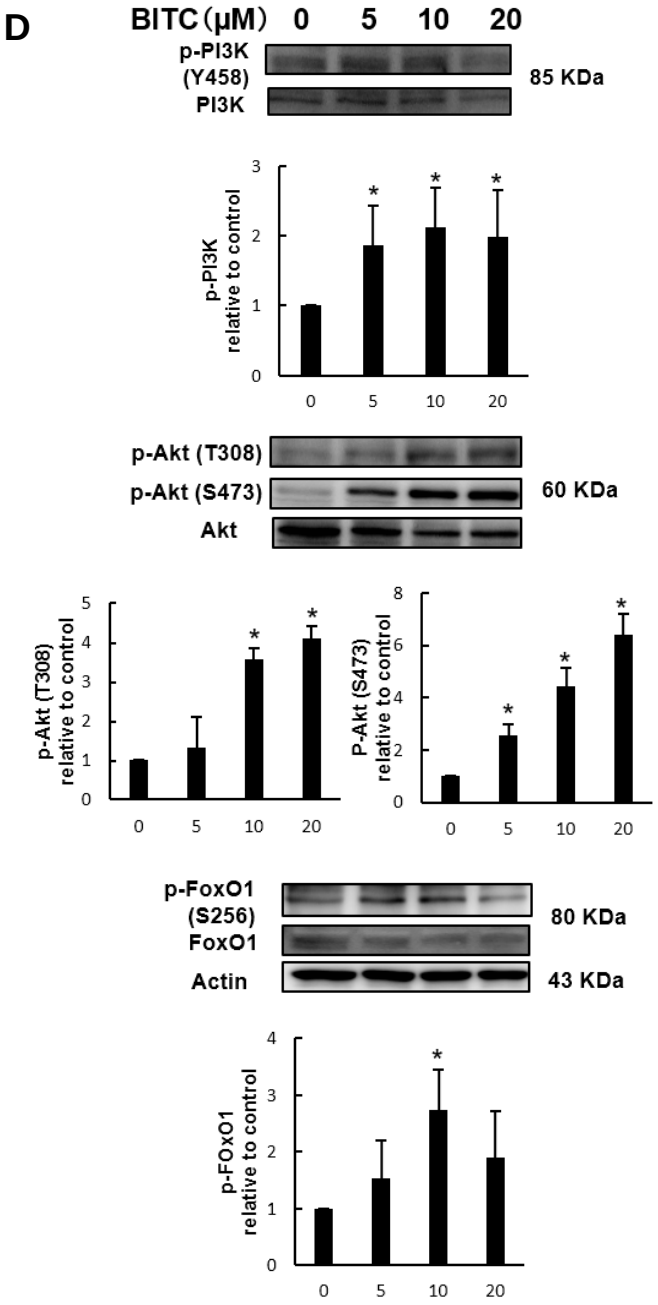
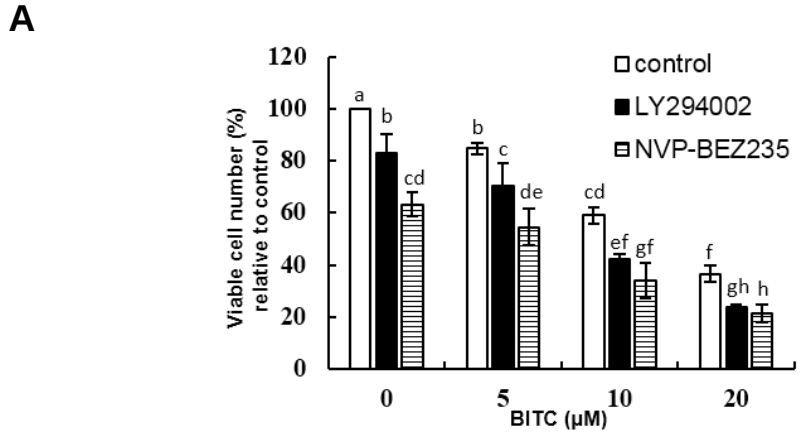


Fig. 2.1 BITC activated the PI3K/Akt/FoxO survival pathway

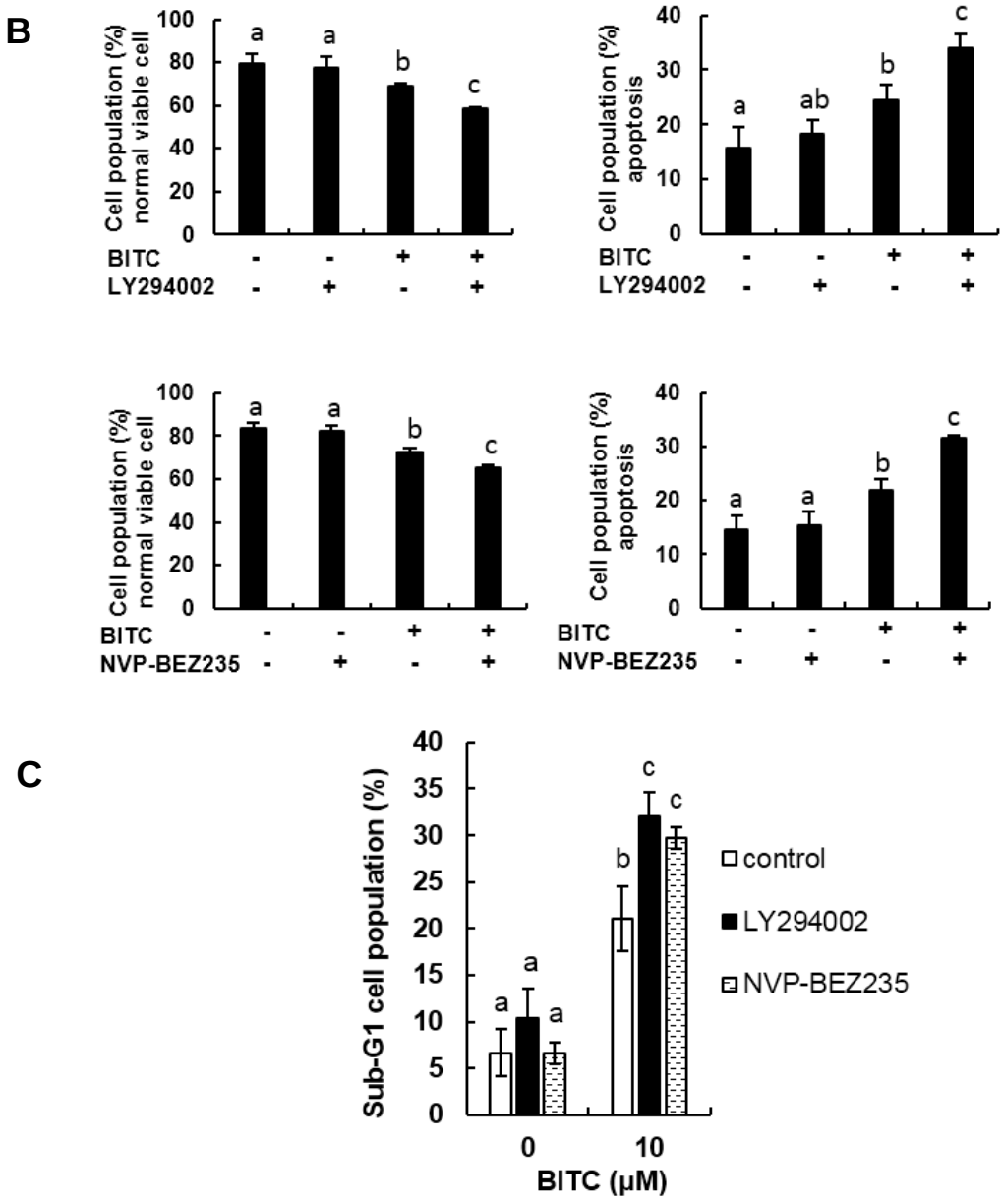
Fig. 2.1 BITC activated the PI3K/Akt/FoxO survival pathway. (A) Antiproliferative effect of BITC in HCT-116 human colorectal cancer cells. HCT-116 cells were treated with the indicated concentrations of BITC for 48 h and cell viability was determined by trypan blue dye exclusion assay. Effects of BITC on the expressions of the PI3K/Akt/FoxO signaling proteins in HCT-116 (B), HT-29 (C) and DLD-1 (D) cells. HCT-116, HT-29 and DLD-1 cells were treated with the indicated concentration of BITC for 1 h. Western blot analysis was performed for phosphorylated and total proteins of IR, PI3K, Akt and FoxO1 as well as actin. The values represent means ± S.D. of three separate experiments (*$P < 0.05$ compared with control; Student's t-test).

2.3.2 Enhancement of antiproliferative effect of BITC by the PI3K inhibitors

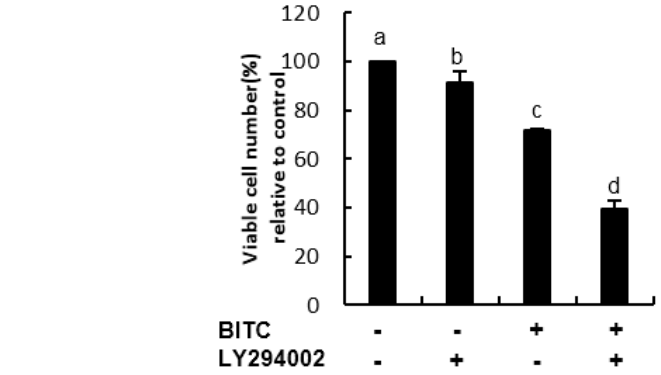
We examined whether the antiproliferative effect of BITC is enhanced by the inhibition of PI3K. A PI3K inhibitor, LY294002, and a dual PI3K/mTOR inhibitor, NVP-BEZ235, were utilized in combination with the BITC treatment. As shown in Fig. 2.2A, LY294002 and NVP-BEZ235 significantly enhanced the BITC-induced antiproliferation with the increasing concentration of BITC, as well as in HT-29 (D) and DLD-1 (F) by LY294002 only. We next clarified the mechanism underlying the enhancement of the BITC-induced antiproliferation by the PI3K inhibitors, LY294002 and NVP-BEZ235. As shown in Figs. 2.2B and 2.2C, both LY294002 and NVP-BEZ235 significantly enhanced the apoptosis induction by BITC, whereas they alone had



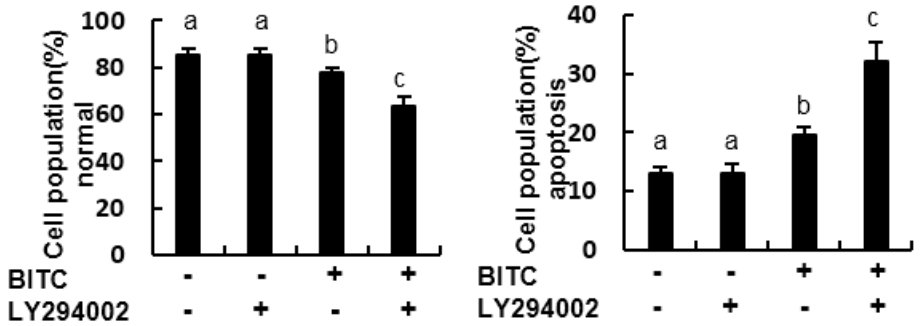
no effects, suggesting that the effect of these inhibitors is synergistic. LY294002 only showed the similar effects on BITC-induced apoptosis in HT-29 (E) and DLD-1 (G) cells. Consistently, as shown in Fig. 2.2C, 10 μM LY294002 and 200 nM NVP-BEZ235 significantly potentiated the increased ratio of the Sub-G_1 cell population by BITC, whereas they had no effects on the ratio of other cell cycle populations, such as G_1, S and G_2/M.



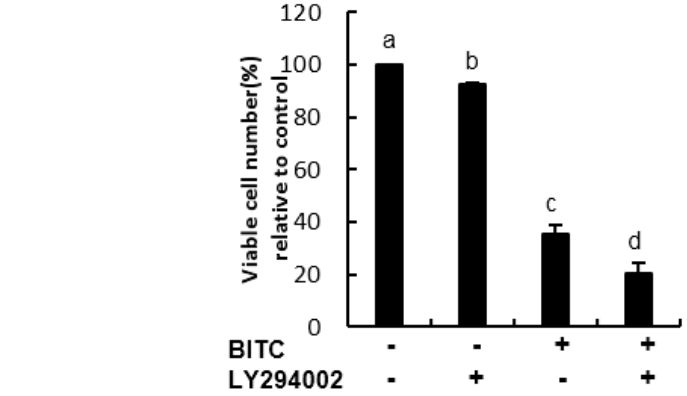
D


Viable cell number(%)
relative to control
120
100
80
60
40
20
0
a
b
c
d
BITC - - + +
LY294002 - + - +

E


Cell population(%)
normal
100
80
60
40
20
0
a a b c
BITC - - + +
LY294002 - + - +
Cell population(%)
apoptosis
40
30
20
10
0
a a b c
BITC - - + +
LY294002 - + - +

F


Viable cell number(%)
relative to control
120
100
80
60
40
20
0
a
b
c
d
BITC - - + +
LY294002 - + - +

G

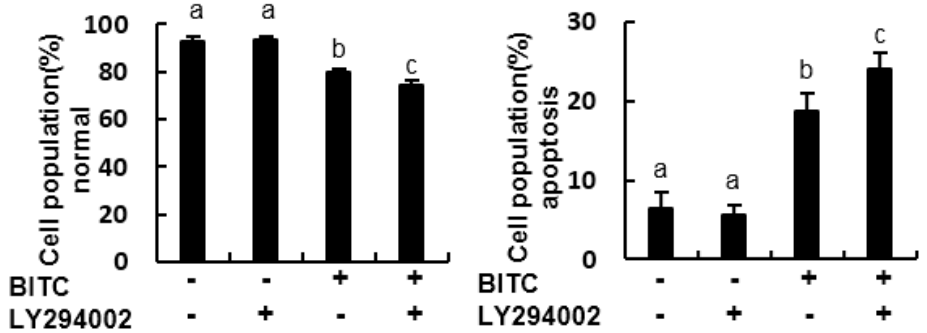
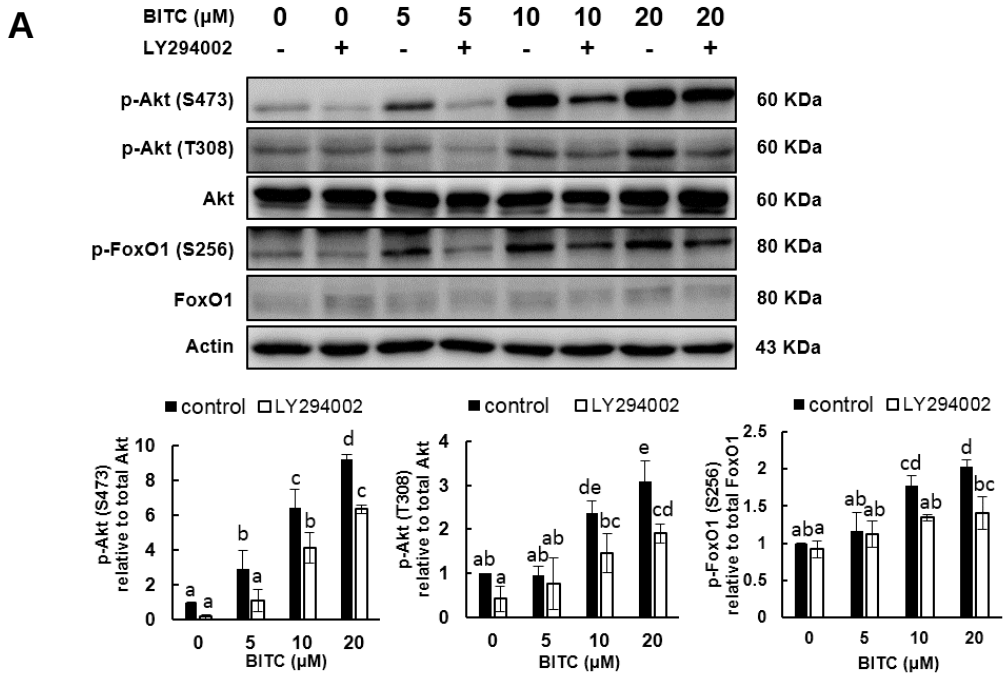

Cell population(%)
normal
100
80
60
40
20
0
a a b c
BITC - - + +
LY294002 - + - +
Cell population(%)
apoptosis
30
20
10
0
a a b c
BITC - - + +
LY294002 - + - +

Fig. 2.2 PI3K inhibitors enhanced the antiproliferative effect of BITC

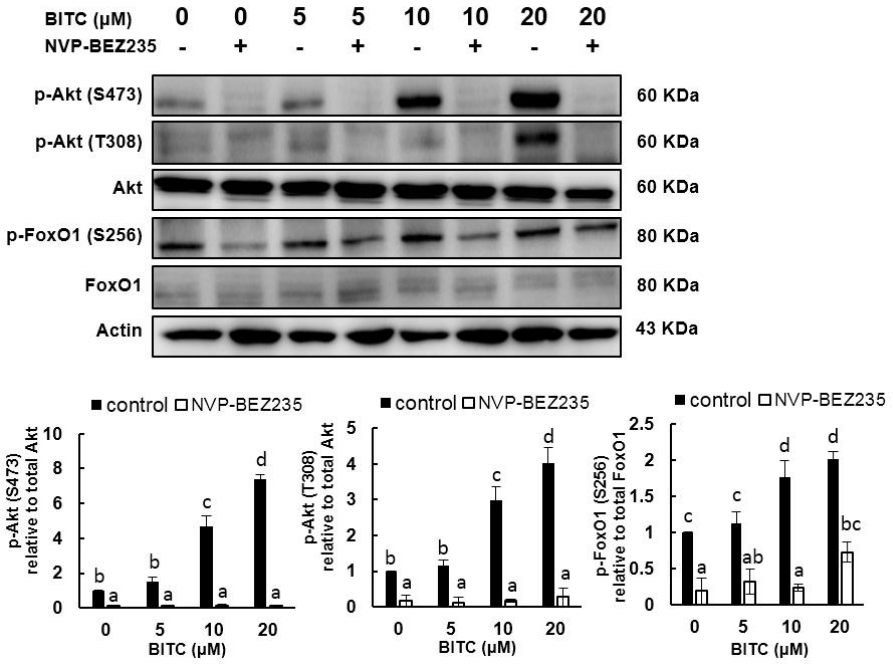
Fig. 2.2 PI3K inhibitors enhanced the antiproliferative effect of BITC. (A, D and F) PI3K inhibitors further decreased the cell viability. Cells were pre-treated with LY294002 (10 μM, A, D and F) and NVP-BEZ235 (200 nM, A) for 1 h and incubated with the indicated concentrations of BITC for 48 h. Cell viability was determined by trypan blue dye exclusion assay. The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$). (B, C, E and G) PI3K inhibitors enhanced the BITC-induced apoptotic cell death. Cells were pre-treated with LY294002 (10 μM, B, E and G) or NVP-BEZ235 (200 nM, B) for 1 h and incubated with or without BITC (10 μM) for 48 h. Apoptosis was detected by an Annexin-V-FLUOS stain kit and analyzed by a Tali™ image-based cytometer. (D) PI3K inhibitors enhanced the BITC-induced appearance of the sub-G1 population. HCT-116 cells were pre-treated with LY294002 (10 μM) or NVP-BEZ235 (200 nM) for 1 h and incubated with or without BITC (10 μM) for 48h. Cell cycle analysis was done by PI staining and Tali™ image-based cytometer. The values represent means ± S.D. of three separate experiments. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$).

2.3.3. Attenuation of the BITC-induced PI3K/Akt/FoxO pathway by the PI3K inhibitors

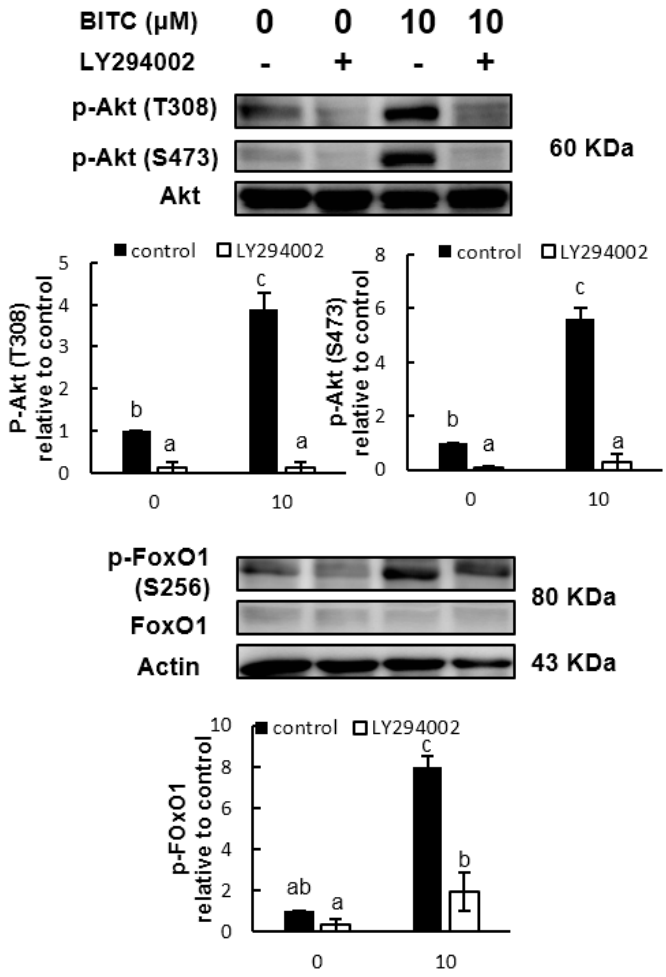
Since the PI3K inhibitors, LY294002 and NVP-BEZ235, significantly enhanced the apoptosis induction by BITC, we next confirmed that these two inhibitors actually attenuate the PI3K/Akt/FoxO survival pathway. As shown in Fig. 2.3A&C&D, LY294002 significantly inhibited the BITC-induced phosphorylation of Akt at both Thr^{308} and Ser^{473} and its downstream target FoxO. Similarly, but more potently, the dual PI3K/$mTORC_2$ inhibitor, NVP-BEZ235, attenuated the BITC-induced phosphorylation of Akt and FoxO (Fig. 2.3B). Compared to the effect of LY294002, NVP-BEZ235 completely diminished the phosphorylation of Akt at Ser^{473} and FoxO, which might be due to the dual inhibitory function against PI3K and $mTORC_2$.



B


BITC (μM) 0 0 5 5 10 10 20 20
NVP-BEZ235 - + - + - + - +
p-Akt (S473)
p-Akt (T308)
Akt
p-FoxO1 (S256)
FoxO1
Actin
60 KDa
60 KDa
60 KDa
80 KDa
80 KDa
43 KDa
control NVP-BEZ235
p-Akt (S473) relative to total Akt
p-Akt (T308) relative to total Akt
p-FoxO1 (S256) relative to total FoxO1
BITC (μM)

C


BITC (μM) 0 0 10 10
LY294002 - + - +
p-Akt (T308)
p-Akt (S473)
Akt
60 KDa
control LY294002
P-Akt (T308) relative to control
p-Akt (S473) relative to control
p-FoxO1 (S256)
FoxO1
Actin
80 KDa
43 KDa
p-FOxO1 relative to control

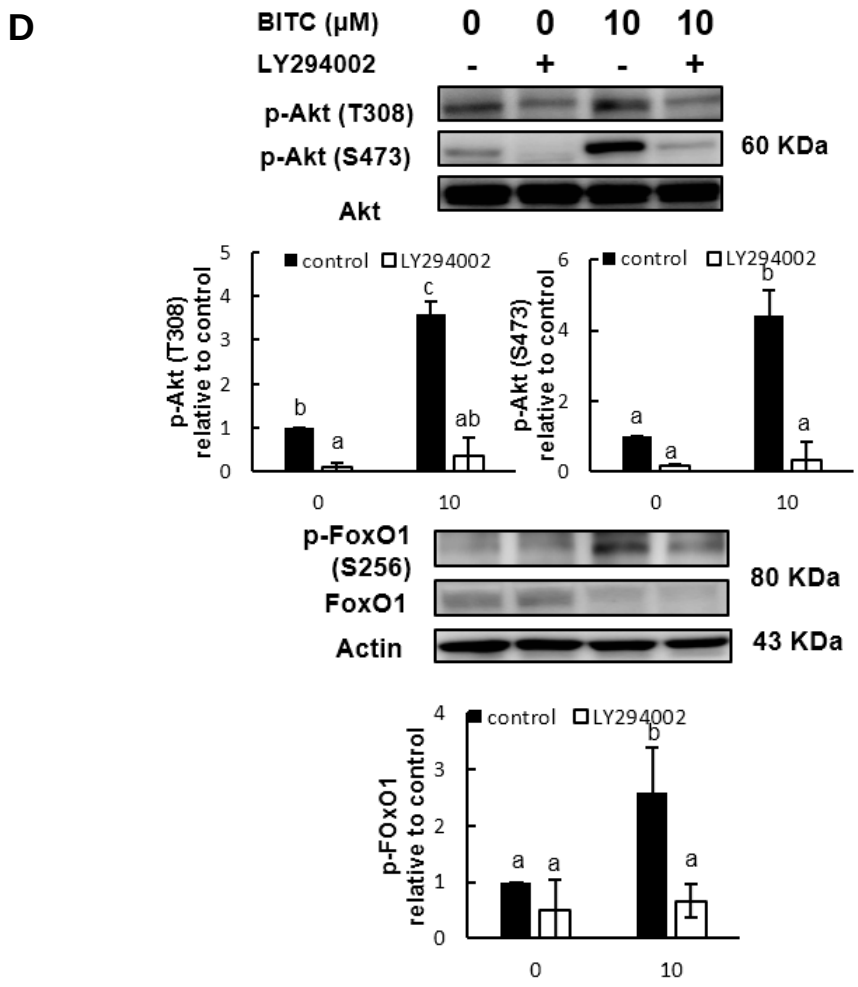


Fig. 2.3. PI3K inhibitors suppressed the BITC-induced phosphorylation of Akt and FoxO

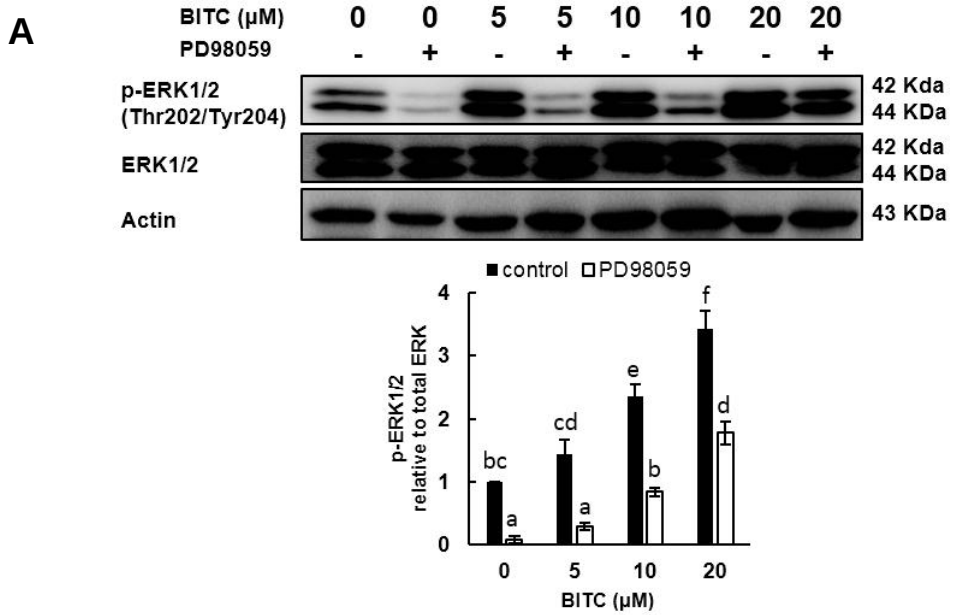
Fig. 2.3. PI3K inhibitors suppressed the BITC-induced phosphorylation of Akt and FoxO. Cells were pre-treated with LY294002 (10 μM, A, C and D) or NVP-BEZ235 (200 nM, B) for 1 h and incubated with the indicated concentrations of BITC for 1 h. Western blot analysis was performed for the phosphorylated and total proteins of Akt and FoxO1 as well as actin. The values represent means ± S.D. of three separate

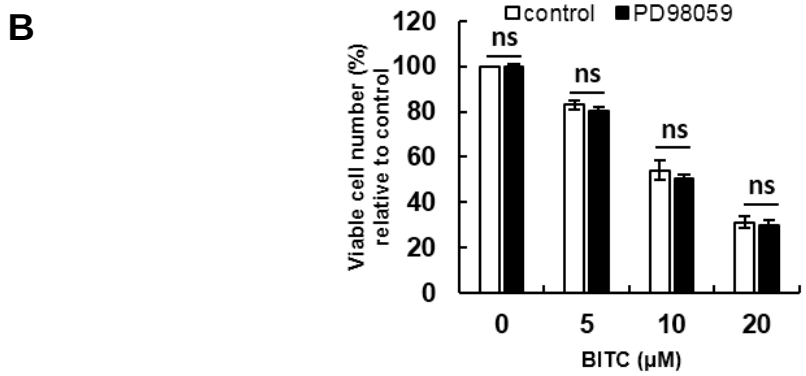
experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD).

2.3.4. Inhibition of MEK/ERK pathway failed to enhance antiproliferation by BITC

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities (Saltiel and Kahn, 2001). Contrast to other MAPKs but similar to the PI3K/Akt pathway, the MEK/ERK signaling cascade plays a significant role cell growth and differentiation (Kim and Choi, 2010; Zhang and Liu, 2002), which is activated by receptor tyrosine kinases (RTK), including insulin receptor (Boucher et al., 2014; Saltiel and Kahn, 2001; Zhang et al., 2011). Since BITC significantly enhanced the phosphorylation of IRβ, we examined whether the ERK pathway is activated by BITC and modulate the BITC-induced antiproliferation. As shown in Figs. 2.4A, BITC significantly enhanced the phosphorylation of ERK and PD98059, a MEK inhibitor, significantly inhibited the enhanced p-ERK level. However, co-treatment of BITC with PD98059 failed to affect the BITC-induced antiproliferation (Fig. 2.4B and 2.4C). Consistently, the treatment of PD98059 had no effect on the ratio of the apoptosis population as well as normal cell population.

Collectively, the BITC-enhanced ERK signaling pathway could be ruled out in the negative regulation of the BITC-induced antiproliferation.





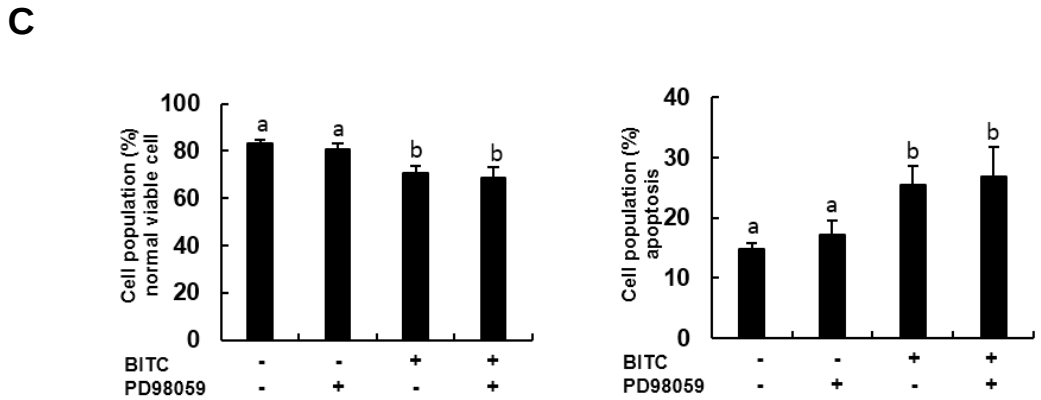
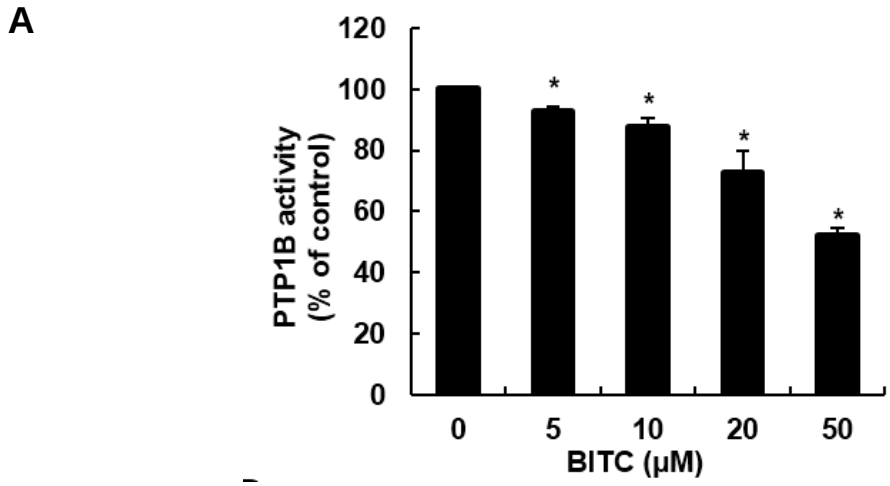


Fig. 2.4 Inhibition of MEK/ERK pathway failed to enhance antiproliferation by BITC

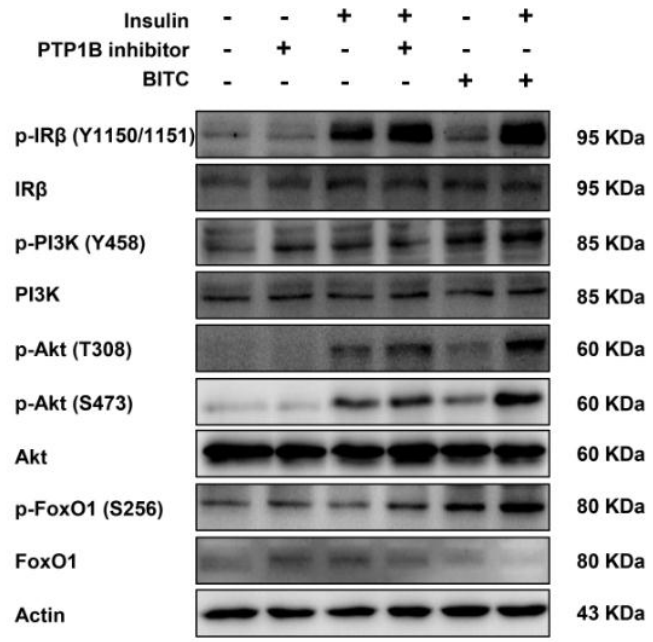
Fig 2.4 Inhibition of MEK/ERK pathway failed to enhance antiproliferation by BITC. (A) PD98059 attenuated the BITC-induced phosphorylation of ERK. HCT-116 cells were pre-treated with PD98059 (10 μM) for 1 h and incubated with the indicated concentrations of BITC for 1 h. Western blot analysis was performed for the phosphorylated and total proteins of ERK as well as actin. The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD). (B) A MEK inhibitor failed to enhance BITC-induced antiproliferation. HCT-116 cells were pre-treated with or without PD98059 (10 μM) for 1 h and incubated with the indicated concentrations of BITC for 48 h. Cell viability was determined by trypan blue dye exclusion assay. The values represent means ± S.D. of three separate experiments (*$P < 0.05$ compared with control; Student's t-test). (C) PD98059 failed to enhance the BITC-induced apoptosis. HCT-116 cells were treated with PD98059 (10 μM) for 1 h and incubated with or without BITC (10 μM) for 48 h. Apoptosis was detected by an Annexin-V-FLUOS stain kit and analyzed by a Tali™ image-based cytometer. The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD).

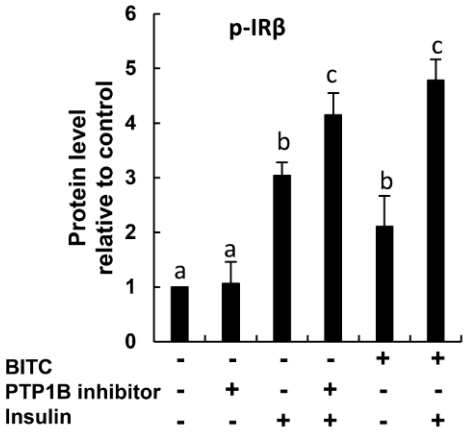
2.3.5. Potentiation of the PI3K/Akt/FoxO pathway by BITC through PTP1B inhibition

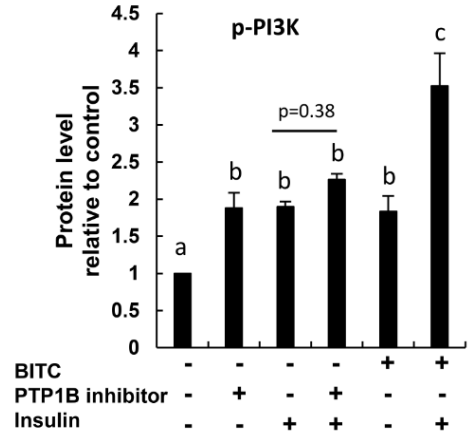
PTP1B has been suggested as an attractive target to improve insulin sensitivity by modulation of the downstream kinase cascade in different cell types (Través et al., 2014). As shown in Fig. 2.4A, BITC inhibited the human recombinant PTP1B enzyme activity in a dose-dependent manner. Consistently, BITC potentiated the phosphorylation levels of IRβ, PI3K and Akt at Ser^{473} not only at the basal level, but also at the insulin-enhanced level (Figs. 2.4B). This tendency is quite similar to that of PTP1B inhibitor XXII, suggesting that BITC enhanced the PI3K/Akt/FoxO pathway by a mechanism similar to that of the PTP1B inhibitor.



B


Insulin - - + + - +
PTP1B inhibitor - + - + - -
BITC - - - - + +
p-IRβ (Y1150/1151) 95 KDa
IRβ 95 KDa
p-PI3K (Y458) 85 KDa
PI3K 85 KDa
p-Akt (T308) 60 KDa
p-Akt (S473) 60 KDa
Akt 60 KDa
p-FoxO1 (S256) 80 KDa
FoxO1 80 KDa
Actin 43 KDa


p-IRβ
Protein level relative to control
0 1 2 3 4 5 6
a a b c b c
BITC - - - - + +
PTP1B inhibitor - + - + - -
Insulin - - + + - +


p-PI3K
Protein level relative to control
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
p=0.38
a b b b b c
BITC - - - - + +
PTP1B inhibitor - + - + - -
Insulin - - + + - +

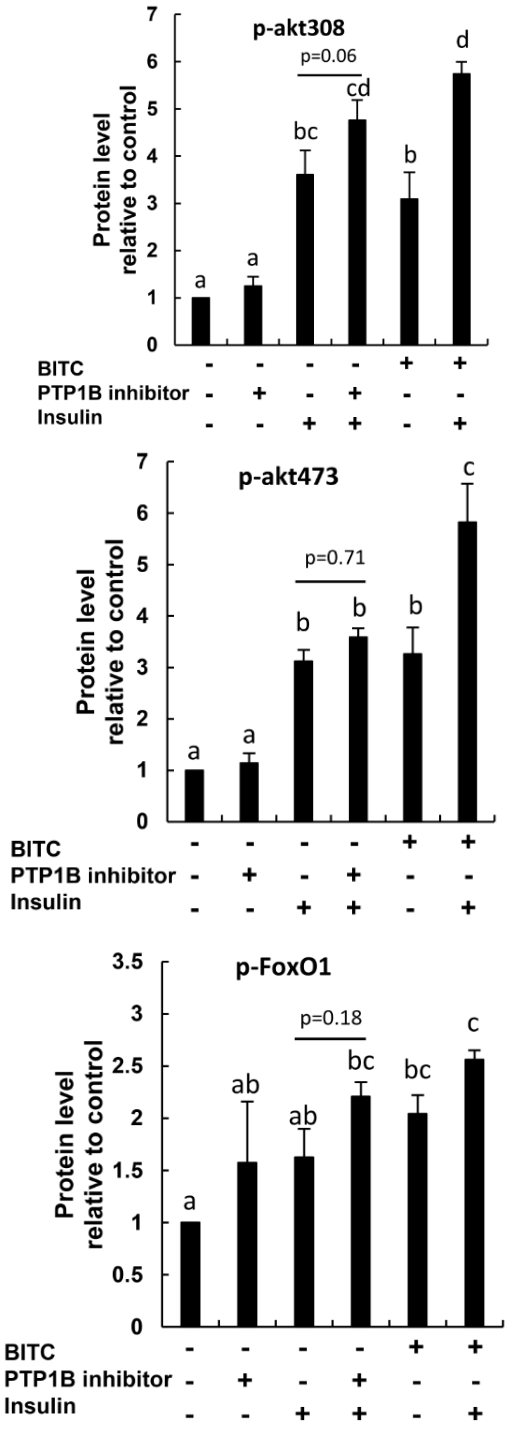

p-akt308
p=0.06
Protein level relative to control
a
a
bc
cd
b
d
BITC
PTP1B inhibitor
Insulin
p-akt473
p=0.71
Protein level relative to control
a
a
b
b
b
c
BITC
PTP1B inhibitor
Insulin
p-FoxO1
p=0.18
Protein level relative to control
a
ab
ab
bc
bc
c
BITC
PTP1B inhibitor
Insulin

Fig. 2.5. BITC potentiated the activation of the PI3K/Akt/FoxO pathway by PTP1B inhibition

Fig. 2.5. BITC potentiated the activation of the PI3K/Akt/FoxO pathway by PTP1B inhibition. (A) BITC inhibited PTP1B enzymatic activity *in vitro*. Human recombinant PTP1B was incubated with the indicated concentrations of BITC and glutathione (1 mM) for 30 min at 37°C in the dark, then incubated with reaction solution in the dark at 37°C for 10 mins. After adding NaOH (1 N) to stop the reaction, absorbance was measured by a microplate spectrophotometer. The values represent means ± S.D. of three separate experiments (*$P < 0.05$ compared with control; Student's t-test). (B) BITC enhanced the insulin-induced phosphorylation of PI3K/Akt/FoxO signaling proteins. HCT-116 cells were pre-treated with PTP1B inhibitor (20 μM) or BITC (10 μM) for 1 h and incubated with or without insulin (200 nM) for 10 min. Western blot analysis was performed for the phosphorylated and total proteins of IR, PI3K, Akt and FoxO1 as well as actin. The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD)

2.4. Discussion

As already mentioned, BITC has been shown to have antiproliferative properties against various cell lines including colorectal cancer cells (Abe et al., 2014; Sakai et al., 2012). Consistent with these reports, we also observed that BITC significantly inhibited the viability of human colorectal cancer HCT-116 cells (Fig. 2.1A). At the same time, BITC significantly potentiated the phosphorylation of the member proteins in the PI3K/Akt/FoxO pathway. BITC not only enhanced the phosphorylation of IRβ, but also initiated a cascade of phosphorylation events, resulting in the phosphorylation of PI3K and Akt at Thr^{308} (Fig. 2.1B). BITC also enhanced the level of p-Akt^{S473}, suggesting that Akt is fully activated and led to the phosphorylation of its downstream target, FoxO1. BITC enhanced PI3K/Akt/FoxO not only in HCT-116 cells, but also in another two human colorectal cancer cell line, HT-29 and DLD-1 (Fig. 2.2C and 2.2D). Previous studies showed that the treatment of BITC for 24 h or the longer period could decrease the phosphorylation levels of PI3K and Akt in HT29 cells (Lai et al., 2010) as well as in human pancreatic cancer cell lines (Boreddy et al., 2011), whereas the effect of its incubation for less than a few hours remained to be determined. To the best of our knowledge, this is the first report showing the activation of the IRβ/PI3K/Akt/FoxO axis by BITC in human colorectal cancer cells.

The PI3K/Akt/FoxO pathway is one of the most famous cell survival pathways that negatively regulate the anti-proliferation and apoptosis induction by anti-tumor agents (Jian et al., 2015). It has been reported that anti-tumor agents inhibit cell proliferation by induction of apoptosis through inhibition of the PI3K/Akt/FoxO pathway in human pancreatic (Boreddy et al., 2011) and colorectal cancer cells (Luo et al., 2013), in which the phosphorylated proteins of PI3K, Akt and FoxO are highly expressed. The fact that BITC enhanced the PI3K/Akt/FoxO pathway (Fig. 2.1B, 2.1C and 2.2D) led to the hypothesis that the PI3K/Akt/FoxO survival pathway is not involved in the antiproliferation mechanism, but rather contributes to the resistance against BITC.

This idea was supported by the observation that the antiproliferation by BITC was significantly enhanced by the PI3K inhibitors at a non-cytotoxic concentration (Fig. 2.2A, 2.2D and 2.2F), but sufficient for attenuation of the Akt phosphorylation (Figs. 2.3A, 2.3B, 2.3C and 2.3D). Furthermore, the combination with the PI3K inhibitors synergistically enhanced the BITC-induced apoptosis in HCT-116, HT-29 and DLD-1 cells (Figs. 2.2B, 2.2E and 2.2G). A similar result was also obtained by a cell cycle analysis (Fig. 2.2C). These results suggested that the inhibition of the PI3K/Akt/FoxO pathway might contribute to enhancement of the apoptosis-inducing signaling in human colorectal cancer cells. Another essential signaling pathway branched from IR is the MAPK/ extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling cascade, which also plays a significant role in cell growth and differentiation (Kim and Choi, 2010; Zhang and Liu, 2002). Although BITC enhanced the phosphorylation of ERK in HCT-116 cell lines, the MEK inhibitor, PD98059, had no effect on the antiproliferation and apoptosis induction by BITC (Fig. 2.4B and 2.4C), suggesting that the ERK signaling cascade could be ruled out in the mechanism of the BITC resistance.

The endoplasmic reticulum-targeted protein tyrosine phosphatase PTP1B is particularly essential in the IR regulation due to its ability to dephosphorylate the phosphorylated IR, thereby switching off insulin signaling. PTP1B regulates a wide variety of cellular processes including cell growth, cell proliferation and apoptosis/survival decisions (Koren and Fantus, 2007). Mice lacking the PTP1B enzyme activity exhibit an enhanced insulin sensitivity, attributable to an increased IR phosphorylation in the liver and muscle (Elchebly et al., 1999). We demonstrated that BITC dose-dependently inhibited the human recombinant PTP1B activity *in vitro* (Fig. 2.5A). Consistently, BITC as well as the PTP1B inhibitor enhanced the phosphorylation of the downstream targets (Fig. 2.5B). These results strongly suggested that BITC might sensitize not only the basal activation, but also the insulin-

induced activation of the PI3K/Akt/FoxO pathway, possibly through the PTP1B inhibition.

In conclusion, we demonstrated the negative regulating role of the PI3K/Akt/FoxO pathway in antiproliferation induced by BITC in human colorectal cancer cells. Inhibitory experiments using the PI3K inhibitors at a non-toxic concentration indicated that inhibition of the PI3K/Akt/FoxO pathway synergistically ameliorates the apoptotic cell death induced by BITC. The present results provide evidence that the combination with inhibitors of the PI3K/Akt/FoxO survival pathway is a promising therapeutic strategy to overcome resistance against food-derived anticancer compounds. Future efforts will be concerned with further understanding the signaling pathway of the apoptosis induction as well as the in vivo significance of the ameliorating effect of the PI3K inhibitors on several rodent models.

CHAPTER 3

A link between benzyl isothiocyanate-induced autophagy and Nrf2/Keap1 regulation in human colon cancer cells

3.1 Introduction

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2)-kelch-like ECH-associated protein 1 (Keap1)-antioxidant response element (ARE) pathway plays a pivotal role in the inducible expression of cytoprotective genes in response to oxidative stress, environmental xenobiotics, and toxic chemicals.(Nakamura and Miyoshi, 2010) Keap1-Cul3 E3 ubiquitin ligase complex mediates the proteasomal degradation of Nrf2 under basal conditions, whereas, under stress conditions, Nrf2 is translocated into nucleus in a Keap1-dependent or -independent manner, followed by transcriptional activation of ARE genes. Oxidative stress and electrophilic chemicals specifically target reactive cysteine residues of Keap1 and lead to release of Nrf2 from the Nrf2-Keap1-Cul3 complex through its conformational change and thus enhanced Nrf2 nuclear translocation (Keap1-dependent canonical pathway).(Qin and Hou, 2016)

Autophagy is a physiological pathway for lysosomal degradation and recirculation in which cellular components, misfolded proteins, and damaged organelles are delivered to double-membrane vesicles called autophagosomes.(Sui et al., 2011) Autophagy is also induced by a variety of stresses, including oxidative stress, ER stress, pathogens, or nutrient deprivation to defend against them and thus preserves cellular homeostasis. Selective substrate adaptor proteins, such as p62/sequestosome 1(SQSTM1), have been shown to facilitate degradation of specific proteins through autophagy,(Ichimura et al., 2008) in addition to its non-selective degradation role. p62 binds directly to microtubule-associated protein 1 light chain 3 (LC3), a representative marker of the autophagosome, that is cleaved (LC3-I) and conjugated to phosphatidylethanolamine (LC3-II).(Pankiv et al., 2007) p62 interacts with ubiquitylated protein aggregates and delivers them to the autophagosomes,(Jiang et al., 2015) indicating an important role for

p62 in autophagic protein degradation. Although the Keap1-dependent canonical Nrf2 regulation is regarded as the primary mode of action for cytoprotection, p62 also regulates the activation of Nrf2 signaling pathway by Keap1 binding and transferring into the autophagosomes for degradation in an autophagy-dependent manner (noncanonical pathway).(Ma, 2013; Wasik et al., 2017)

Isothiocyanates (ITCs), derived from various cruciferous vegetables, are regarded as potential preventive agents against carcinogenesis, because they are capable of up-regulating the xenotiotic-detoxifying enzymes, inducing apoptosis, and inhibiting cell cycle progression.(Nakamura and Miyoshi, 2010) Benzyl isothiocyanate (BITC), an ITC compound from papaya seeds,(Nakamura et al., 2007a) has been shown not only to inhibit cell proliferation in T lymphocytes,(Miyoshi et al., 2004b) renal proximal tubular cells,(Abe et al., 2012) and colorectal cancer cells,(Abe et al., 2014; Sakai et al., 2012) but also to induce the phase 2 drug-metabolizing enzyme.(Nakamura et al., 2000) BITC has recently been reported to induce autophagy in human lung cancer cells(Zhang et al., 2017) and human breast cancer cells.(Xiao et al., 2012) Although Keap1 is thought to be the major target for Nrf2 activation by ITCs such as sulforaphane, the regulating role of autophagy in BITC-induced Nrf2 activation remains unclear.

In this study, we demonstrated that BITC dose-dependently induced autophagy and p62 expression, concomitantly with Keap1/Nrf2 modulation in human colorectal cancer HCT-116 cells. We also clarified the mediating role of phosphatidylinositide 3-kinase (PI3K) between autophagy induction and Nrf2 activation by BITC.

3.2 Materials and Methods

3.2.1Cell Culture.

HCT-116 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT-116 cells were maintained in DMEM (Dulbecco's modified Eagle's medium, high glucose). All medium were supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown at 37ºC in an atmosphere of 95 % O_2 and 5 % CO_2.

3.2.2 Chemicals and Antibodies.

BITC were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Antibodies against LC3B, LAMP1 and Nrf2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against Keap1, P62, actin and horseradish peroxidase-linked anti-rabbit, anti-mouse and anti-goat IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Wortmannin and protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fatal bovine serum (FBS) was purchased from Nichirei Corporation (Tokyo, Japan). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Chemi-Lumi One Super was purchased from Nakalai Tesque Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.2.3 Western Blot Analysis.

See chapter 2.

3.2.4Immunofluorescence.

Cells were incubated in 4% Paraformaldehyde (PFA)/PBS for 30 min. After fixation, cells were incubated in 0.05% TritonX-100/PBS for 10 min and then incubated in 3% BSA/PBS for 1h. Cells were incubated with the primary antibody overnight at 4ºC followed by an appropriate secondary antibody conjugated with FITC. After wash by distilled water, cells were mounted by Fluoroshield Mounting Medium with DAPI and visualized under fluorescence microscope (Keyence).

3.2.5MDC staining.

Cells were washed with ice-cold phosphate-buffered saline without calcium and magnesium (PBS (-)). Autophagic vacuoles were then labeled with dansylcadaverine (MDC) by incubating cells with 0.05 mM MDC at 37^{o}C for 10 min. After incubation, cells were visualized under fluorescence microscope (Keyence).

3.2.6 RNA extraction and RT-PCR.

Total RNA was extracted using Trizol reagent according to the manufacturer's manual and reverse transcribed to cDNA using ReverTra Ace. PCR amplification was then performed with Taq polymerase. Primers used in PCR amplification are as follows: human hemeoxygenase 1 (hHO-1), (F) 5'-AAGATTGCCCAGAAAGCCCTGGAC-3' and (R) 5'-AACTGTCGCCACCAGAAAGCTGAG-3'; human β-actin, (F) 5'-GTCACCCACACTGTGCCCATCTA-3' and (R) 5'-GCAATGCCAGGGTACATGGTGGT -3'. The PCR product were separated on an agarose gel (3%), stained with ethidium bromide, and visualized under UV light. The relative densities of bands were measured using Image J Software Program.

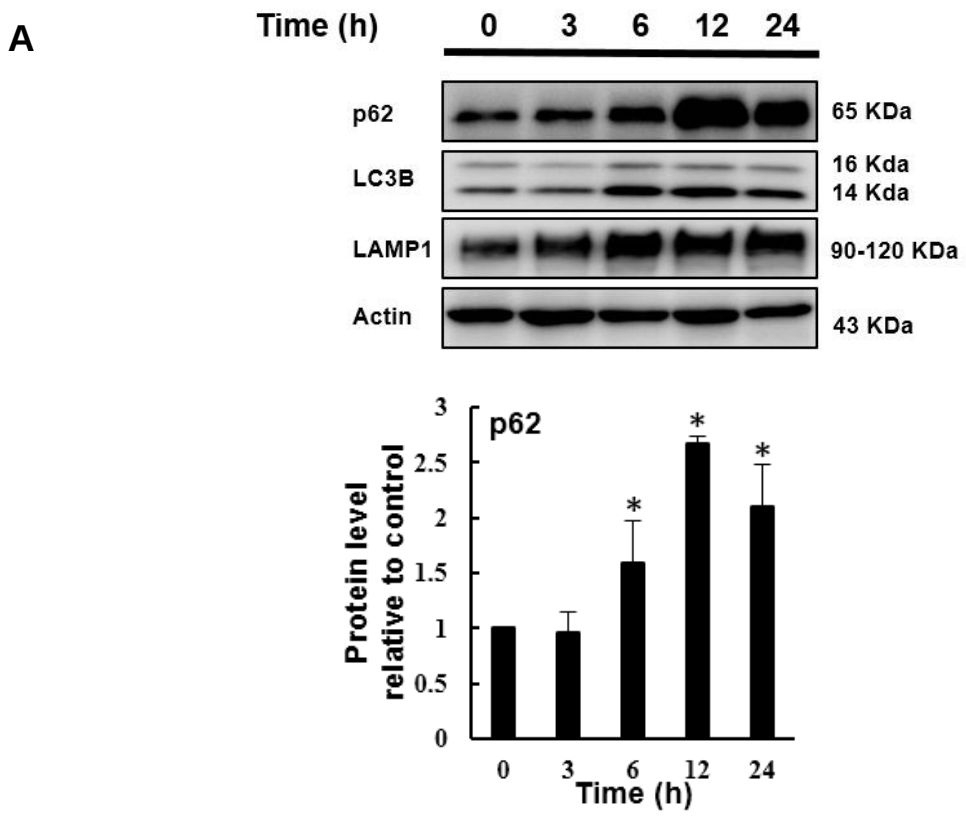
3.2.7 Statistical Analysis.

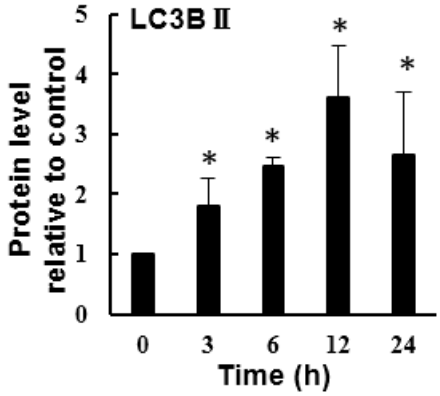
The data are expressed as the means ± standard deviation (S.D.) of at least three independent experiments and were analyzed using Student`s t-test or Tukey test for comparison between groups. *P* values of < 0.05 were considered to be statistically significant.

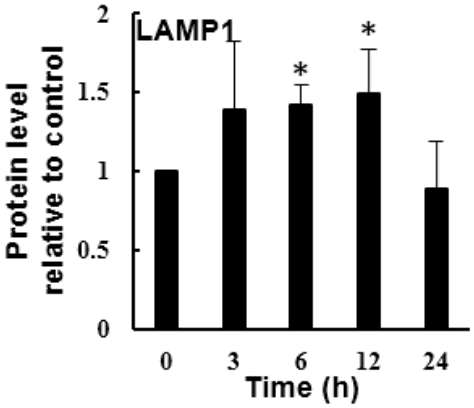
3.3 Results

3.3.1 BITC induced the accumulation of autophagic molecules in HCT-116 cells

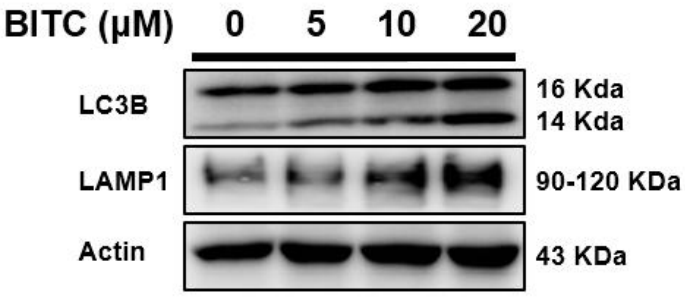
Initial experiments using human colorectal cancer cells showed that BITC enhanced the protein levels of representative molecules of autophagy, including p62, LAMP1 as well as the conversion of LC3BI into LC3BII in a time-dependent manner up to 12 h (Fig. 3.1A). As shown in Figs. 3.1B, BITC dose-dependently enhanced protein levels of LC3BII and LAMP1, which suggested that BITC might induced the formation of autophagosome and lysosome. In addition, due to recent studies that the crosslink between Nrf2-Keap1-ARE axis with autophagy, I also found that BITC concomitantly induced p62 and Nrf2 up-regulation and Keap1 down-regulation. These results suggested that BITC induced not only autophagy, but also Nrf2 pathway activation in HCT-116 cells.

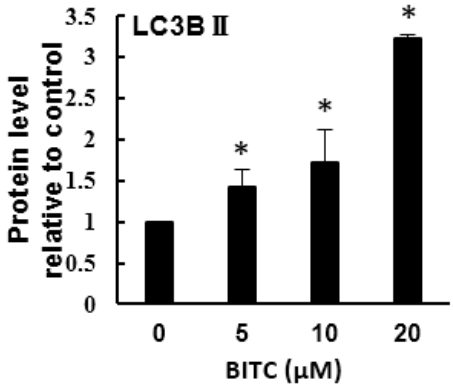


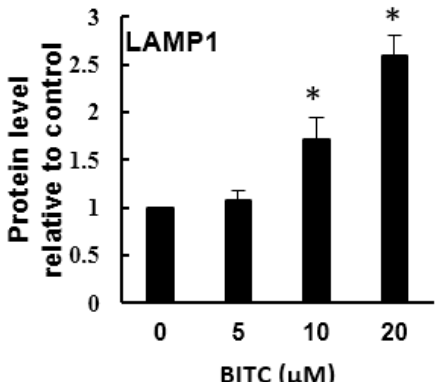

LC3B Ⅱ
Protein level relative to control
0
1
2
3
4
5
0
3
6
12
24
Time (h)
*
*
*
*


LAMP1
Protein level relative to control
0
0.5
1
1.5
2
0
3
6
12
24
Time (h)
*
*
*

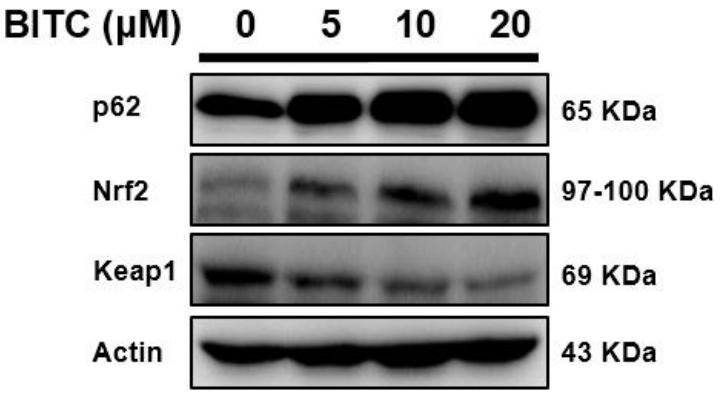
B

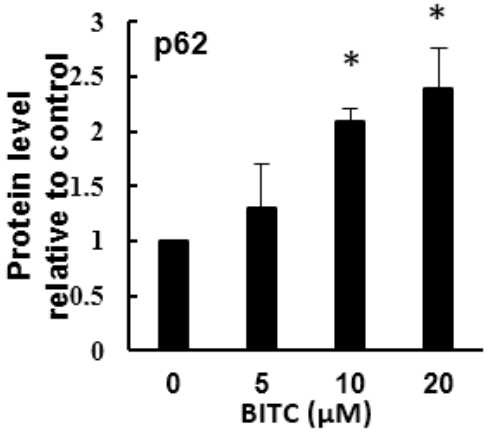

BITC (μM)
0
5
10
20
LC3B
16 Kda
14 Kda
LAMP1
90-120 KDa
Actin
43 KDa

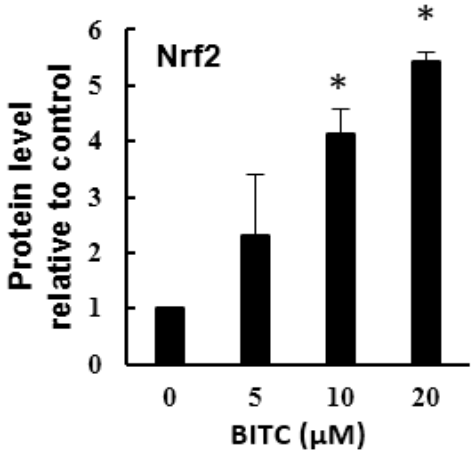

LC3B Ⅱ
Protein level relative to control
0
0.5
1
1.5
2
2.5
3
3.5
0
5
10
20
BITC (μM)
*
*
*
*


LAMP1
Protein level relative to control
3
2.5
2
1.5
1
0.5
0
0
5
10
20
BITC (μM)
*
*

C


BITC (μM) 0 5 10 20
p62
65 KDa
Nrf2
97-100 KDa
Keap1
69 KDa
Actin
43 KDa


p62
Protein level relative to control
3
2.5
2
1.5
1
0.5
0
0
5
10
20
BITC (μM)
*
*


Nrf2
Protein level relative to control
6
5
4
3
2
1
0
0
5
10
20
BITC (μM)
*
*

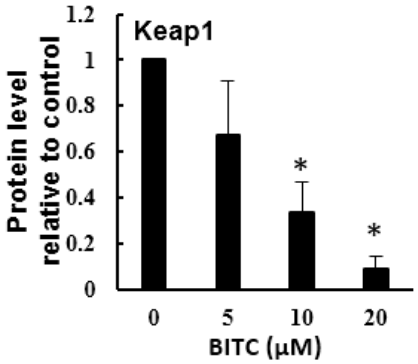


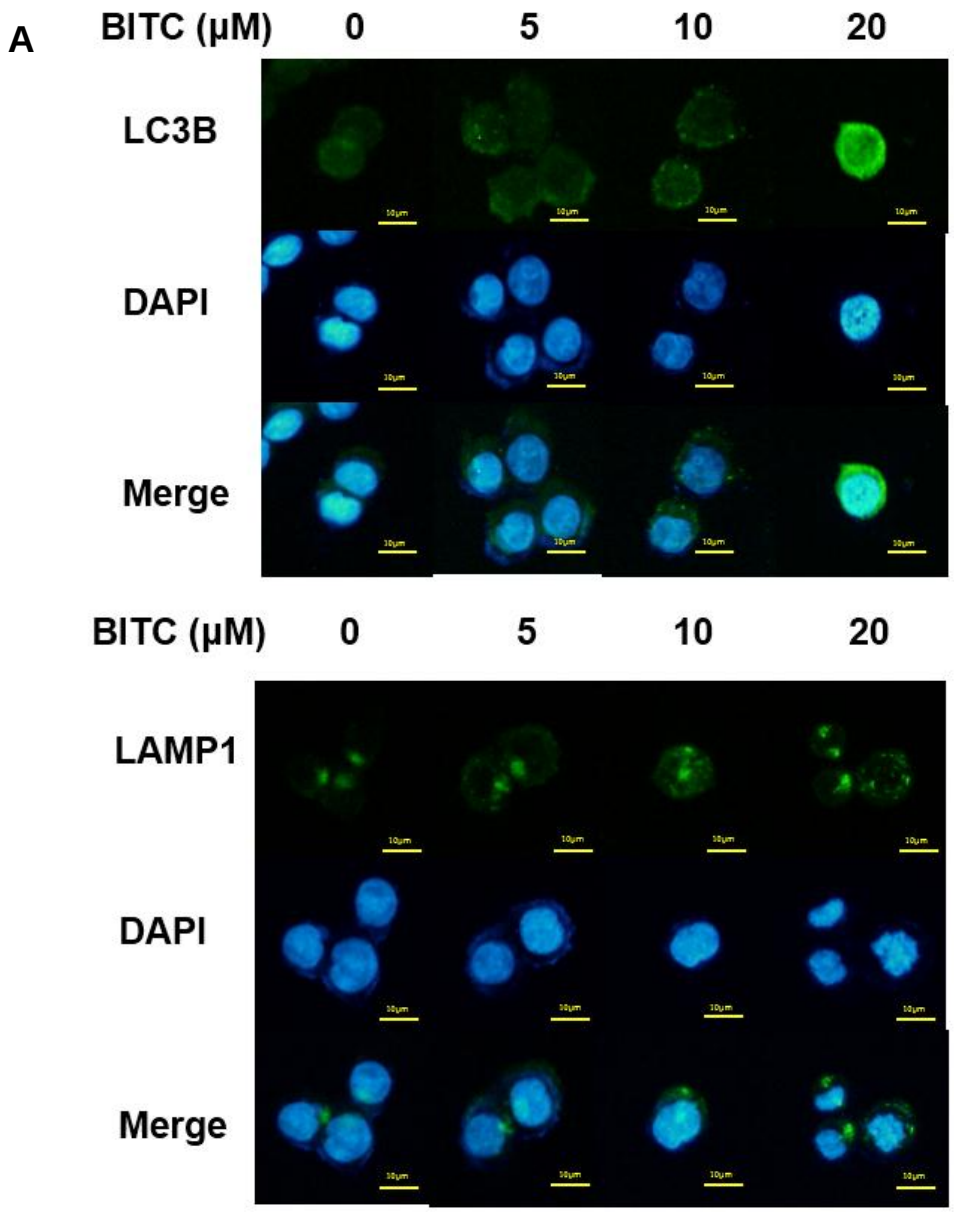
Fig.3.1 BITC enhanced protein levels of representative molecules of autophagy and activated p62-related Nrf2/Keap1 pathway

Fig.3.1 BITC enhanced protein levels of representative molecules of autophagy and activated p62-related Nrf2/Keap1 pathway. (A). Time-dependent induction of auphagic molecules by BITC. HCT-116 cells were treated with the indicated period of 20 μM BITC. Western blot analysis was performed for proteins of p62, LC3B and LAMP1 as well as actin. (B). Induction of autophagic molecules by BITC in HCT-116 cancer cells. HCT-116 cells were treated with the indicated concentrations of BITC for 12 h. Western blot analysis was performed for proteins of LC3B and LAMP1 as well as actin. (C) Activation of p62-Nrf2-Keap1 axis by BITC. HCT-116 cells were treated with the indicated concentrations of BITC for 12 h. Western blot analysis was performed for proteins of p62, Nrf2 and Keap1 as well as actin. The values represent means ± S.D. of three separate experiments (*$P < 0.05$ compared with control; Student's t-test).

3.3.2 BITC induced the accumulation of autophagic compartments in HCT-116 cells

To further examine BITC-induced autophagy, we performed immunostaining with anti-LC3B and anti-LAMP1 antibodies. HCT-116 cells were treated with BITC for 12 h. Immunocytochemistry experiments showed that the incubation of BITC for 12 h resulted in the increment in puncta of LC3B and LAMP1 (Fig. 3.2A) , further supporting the idea that BITC induces the formation of autophagosome and lysosome. The cytosolic MDC staining observed under fluorescence microscope were determined that revealed BITC

induced accumulation of autophagic vacuoles in a dose-dependent manner (Fig. 3.2B).



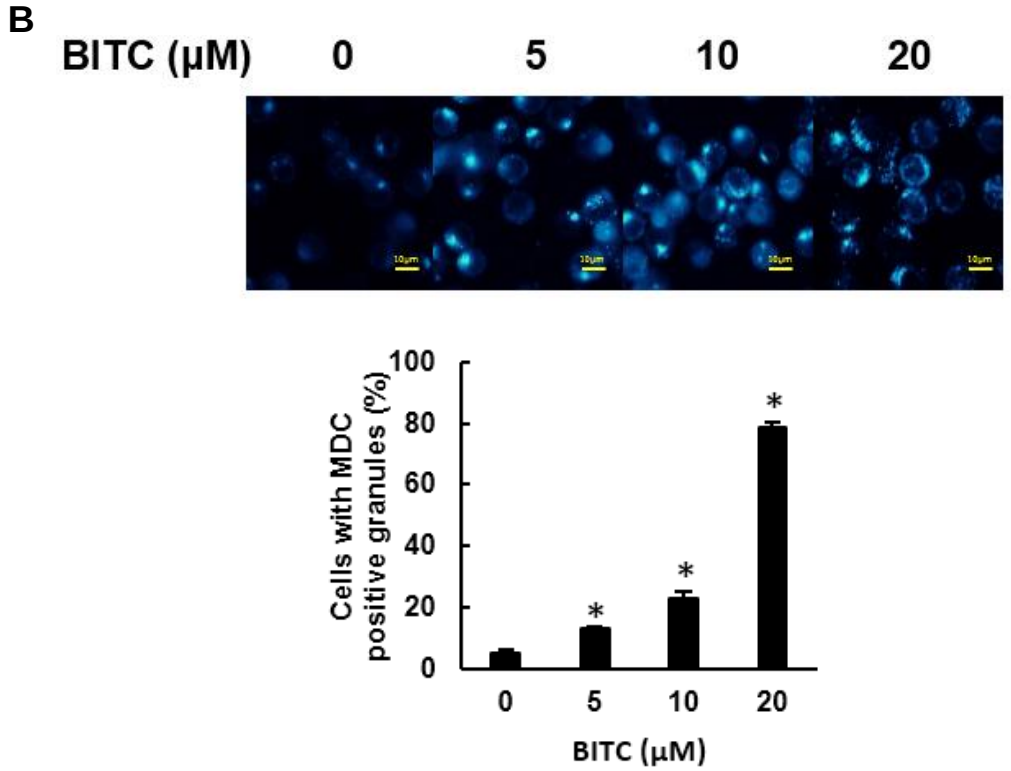
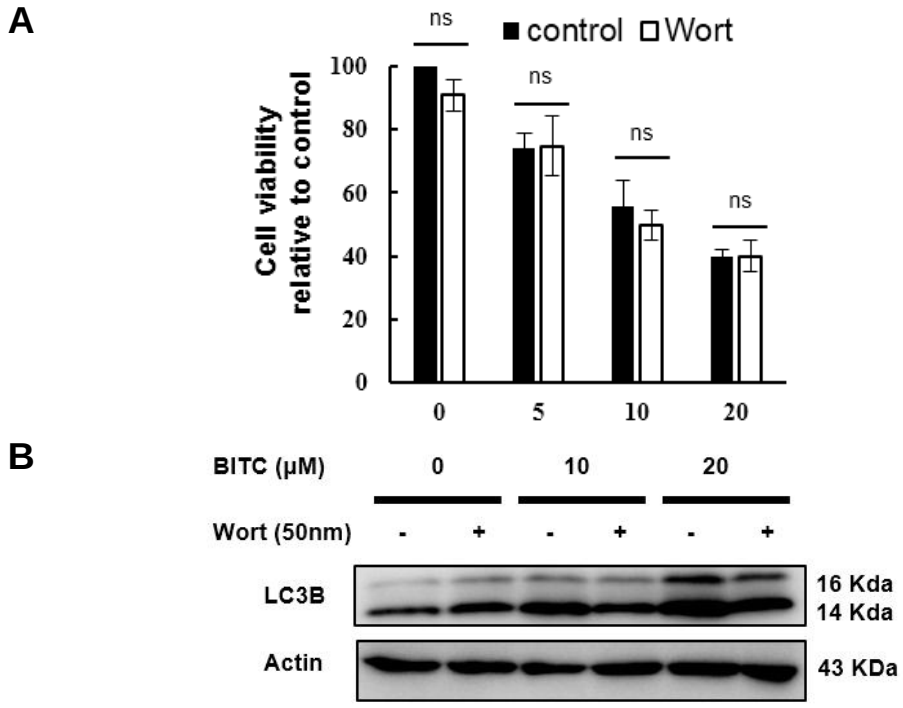


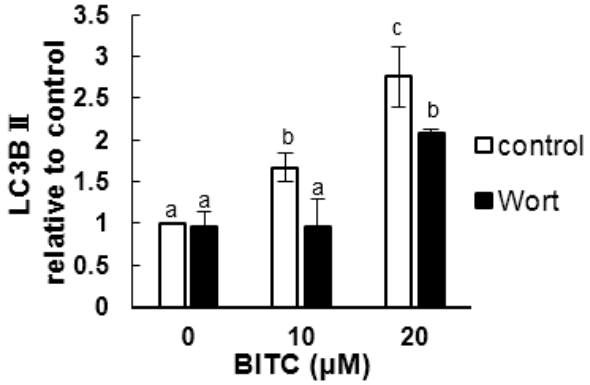
Fig. 3.2 BITC induced formation and accumulation of autophagic compartments in HCT-116 cancer cells

Fig. 3.2 BITC induced formation and accumulation of autophagic compartments in HCT-116 cancer cells. (A). BITC induced formation of autophagome and lysosome. HCT-116 cells were treated with the indicated concentrations of BITC for 12 h. cells were immuno-fluorescently labeled and imaged using a fluorescence microscope. LC3B and LAMP1 represents formation of autophagosome and lysosome, respectively. (B) Induction of autophagic vacuoles by BITC. HCT-116 cells were treated with the indicated concentrations of BITC for 12 h before MDC staining. Cells were visualized under fluorescence microscope. The number of cells with a granular positive MDC staining was counted (a minimum of 100 cells/sample). The values represent means ± S.D. of three separate experiments (*$P < 0.05$ compared with control; Student's t-test).

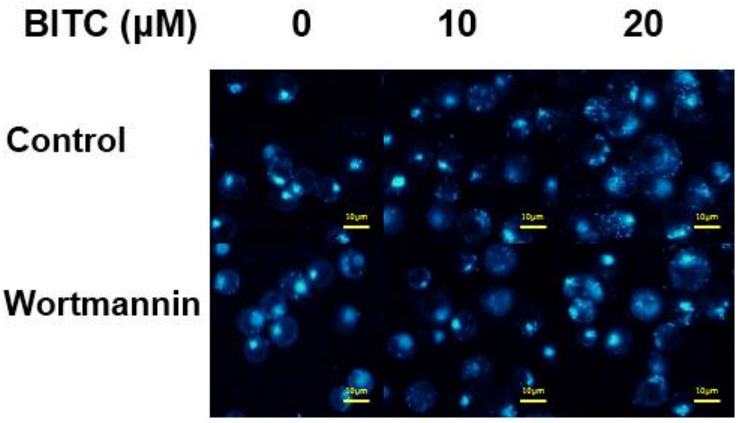
3.3.3 PI3K inhibitor wortmannin attenuated BITC-enhanced conversion of LC3B and autophagic vacuoles in HCT-116 cells.

Class III PI3K (PIK3C3/Vps34) has been strongly implicated in autophagic processes in mammals.(Itakura et al., 2008) Both LC3B and p62 are prerequisite for the biosynthesis of autophagosome, which is also regulated by PI3K.(Kim and Choi, 2010) Wortmannin, a selective PI3K inhibitor, has been reported to possess effects on the inhibition of autophagy in diverse cancer cell lines.(Huang et al., 2016; Zhou et al., 2017; Zhu et al., 2017) As shown in Fig. 3.2B, wortmannin significantly attenuated the BITC-induced conversion of LC3B in a non-cytotoxic concentration (Fig. 3.2A), implying that PI3K is involved in the BITC-induced autophagy in HCT-116 cells. In addition, MDC staining results, as shown in Fig 3.3C, suggested wortmannin significantly attenuated the BITC-induced autophagic vacuoles in HCT-116 cells.




LC3B II
relative to control
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
a
c
b
0
10
20
BITC (μM)
control
Wort

C


BITC (μM)
0
10
20
Control
Wortmannin

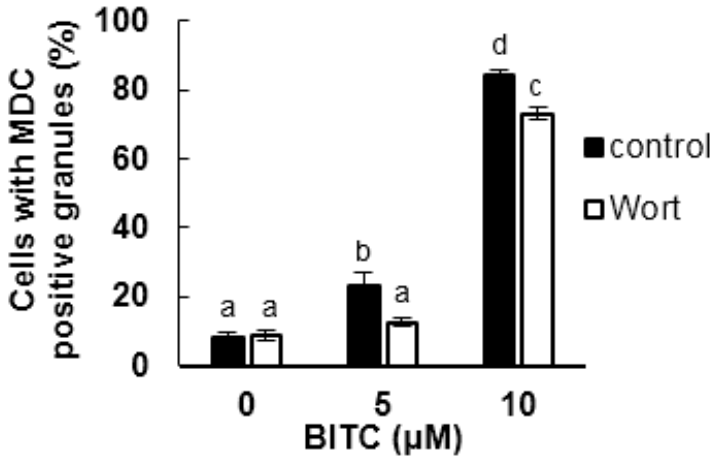

Cells with MDC
positive granules (%)
100
80
60
40
20
0
a
a
b
a
d
c
0
5
10
BITC (μM)
control
Wort

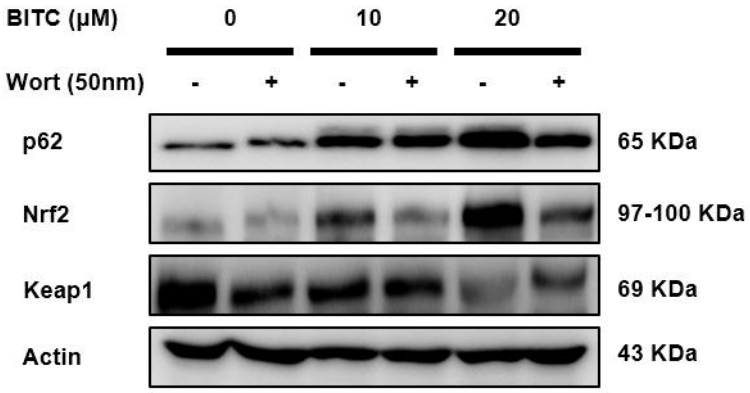
Fig. 3.3 Wortmannin attenuated BITC-induced conversion of LC3B and autophagic vacuoles in HCT-116 cells.

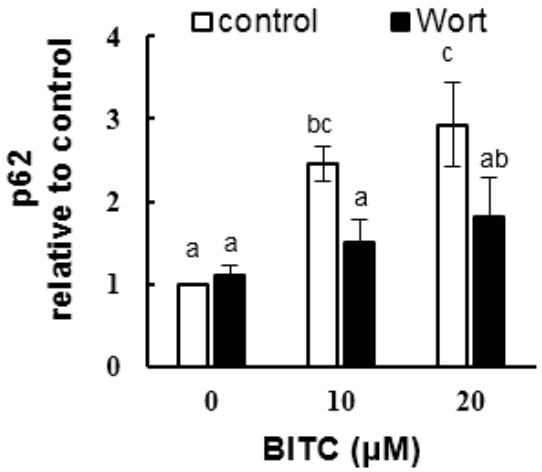
Fig 3.3 Wortmannin attenuated BITC-induced conversion of LC3B and autophagic vacuoles in HCT-116 cells. (A). Wortmannin (50nm) had no effects on cell viability in HCT-116 cells. HCT-116 cells were pre-treated with wortmannin (50 nM) for 1 h and incubated with the indicated concentrations of BITC for 24 h. Cell viability was measured by MTT assay. (B). Wortmannin attenuated BITC-induced autophagic molecules. HCT-116 cells were pre-treated with wortmannin (50 nM) for 1 h and incubated with the indicated concentrations of BITC for 12 h. Western blot analysis was performed for proteins of LC3B as well as actin. (C). Wortmannin attenuated BITC-induced autophagic vacuoles. HCT-116 cells were pre-treated with wortmannin (50 nM) for 1 h and incubated with the indicated concentrations of BITC for 12 h before MDC staining. Cells were visualized under fluorescence microscope. The number of cells with a granular positive MDC staining was counted (a minimum of 100 cells/sample). The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD).

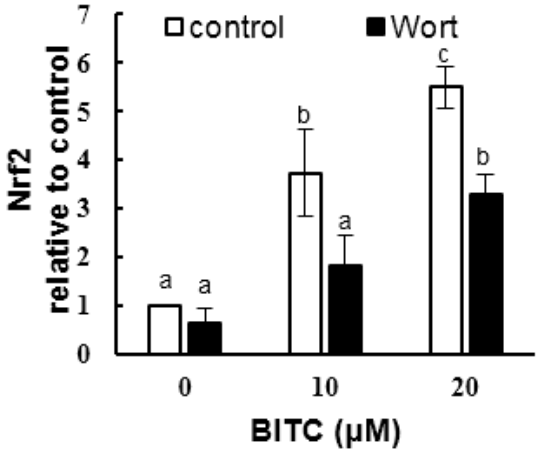
3.3.4 PI3K inhibitor wortmannin attenuated BITC-induced p62-Nrf2-Keap1 pathway in HCT-116 cells.

We next examined whether the PI3K inhibitor could affect the Keap1/Nrf2 pathway. As shown in Fig. 2, wortmannin completely impaired the BITC-induced accumulation of protein levels of p62 and Nrf2, coincided with the full recovery of down-regulated Keap1 (Fig. 3.4A). Furthermore, wortmannin significantly impaired the BITC-induced up-regulation of the gene expression of HO-1, one of the representative Nrf2-regulated genes (Fig. 3.4B). These results suggested that PI3K plays the key role in the autophagy-dependent Nrf2 activation by BITC.

A


BITC (μM)
0
10
20
Wort (50nm)
-
+
-
+
-
+
p62
65 KDa
Nrf2
97-100 KDa
Keap1
69 KDa
Actin
43 KDa


control
Wort
p62
relative to control
0
1
2
3
4
a
a
bc
a
c
ab
0
10
20
BITC (μM)


control
Wort
Nrf2
relative to control
0
1
2
3
4
5
6
7
a
a
b
a
c
b
0
10
20
BITC (μM)

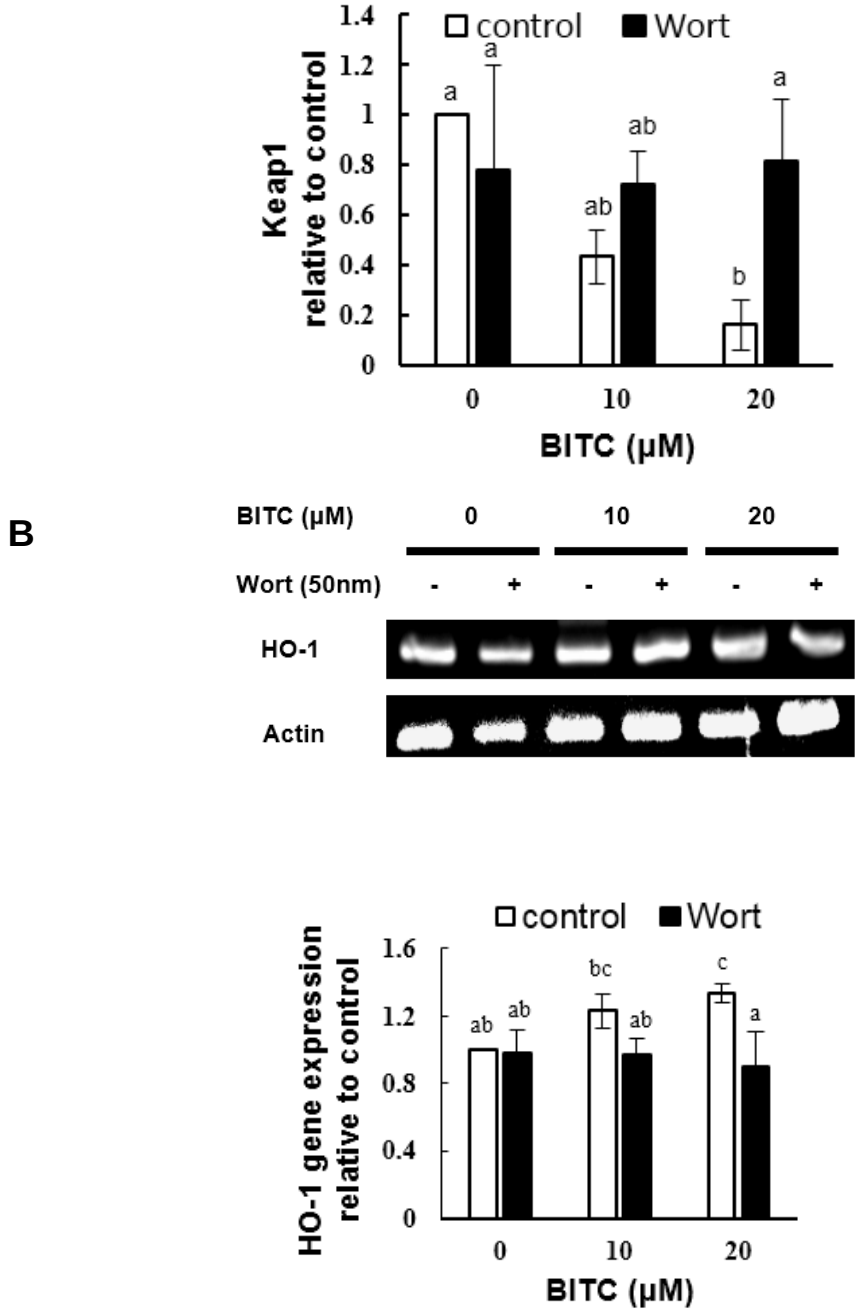


Fig. 3.4 PI3K inhibitor wortmannin attenuated BITC-induced p62-Nrf2-Keap1 pathway in HCT-116 cells

Fig. 3.4 PI3K inhibitor wortmannin attenuated BITC-induced p62-Nrf2-Keap1 pathway in HCT-116 cells. (A). Wortmannin attenuated BITC-induced actication of p62-Nrf2-Keap1 pathway. HCT-116 cells were pre-treated with wortmannin (50 nM) for 1 h and incubated with the indicated concentrations of BITC for 12 h. Western blot analysis was performed for proteins of p62, Nrf2 and Keap1 as well as actin. (B). Wortmannin attenuated BITC-induced HO-1 gene expression. HCT-116 cells were pre-treated with wortmannin (50 nM) for 1 h and incubated with the indicated concentrations of BITC for 12 h. RT-PCR was performed for HO-1 as well as actin. The values represent means ± S.D. of three separate experiments. Different letters above the bars indicate significant differences among treatments for each compound ($P > 0.05$, Tukey's HSD).

Discussion

Autophagy is responsible for scavenging and degrading of damaged organelles, long-lived proteins, for misfolded proteins, which also plays an essential role in eliminating intracellular pathogens to maintain the cellular longevity (Mizushima et al., 2008). Given its critical roles, it is extremely important to investigate its underlying mechanism in cancer cells. In the present study, we found BITC dose-dependently induced accumulation of autophagic molecules (Fig.3.1 B), which reached the maximum level at 12 h. (Fig. 3.1A). Moreover, we morphologically confirmed that BITC induced not only the formation of autophagosome and lysosome (Fig. 3.2A) but also the formation of autophagic vacuoles (Fig. 3.2B). On the other hand, induction of p62 by BITC (Fig. 3.1A) suggested that p62/Nrf2 pathway might be involved in BITC-induced autophagy in HCT-116 cells. Surprisingly, our results indicated that p62-Nrf2-Keap1 axis was activated by a noncanonical pathway (Fig.3.1C).

In general, both LC3B and p62 are necessarily required for the biosynthesis of autophagosome which is regulated by PI3-kinase (Funderburk et al., 2010). Once we found that BITC induced autophagy in HCT-116 cells, we hypothesized that PI3K might be involved in BITC-induced autophagy. The idea was supported by the investigation that PI3K inhibitor wortmannin inhibited the accumulation of LC3B (Fig. 3.3A), autophagic vacuoles (Fig. 3.3B) and p62 (Fig. 3.4A) in a non-toxic concentration (Fig. 3.3A), indicating that PI3K was involved in the mechanism in BITC-induced autophagy. On the other hand, the inhibition of BITC-induced accumulation of p62 by wortmannin also attenuated BITC-induced noncanonical p62-Nrf2-Keap1 pathway (Fig. 3.4 A). The noncanonical Nrf2/Keap1 pathway contributes to the activation of Nrf2, which was also confirmed by the found that inhibition of Nrf2 by equivalent amount of wortmannin resulted in attenuation of BITC-induced HO-1 gene expression (Fig. 3.4 B). These results suggested that BITC triggered autophagic effect in PI3K-associated mechanism through a p62-dependent Nrf2/Keap1 pathway in HCT-116 cells.

In conclusion, we demonstrated that BITC activated the Nrf2 pathway in an autophagy-dependent manner (noncanonical pathway) in human colorectal cancer HCT-116 cancer cells. The present data suggested that PI3K plays an essential role in association between Nrf2/Keap1 pathway and autophagy in HCT-116 cells. Drug resistance often limits the efficacy as well as outcome of chemotherapy. The increasing metabolism and efflux of the drug as well as PI3K mediates resistance to the chemotherapy drugs.(Gottesman et al., 2002) The PI3K-mediated pathway is frequently activated(Boreddy et al., 2011; Fahy et al., 2003) and influences cell growth, survival and drug resistance(Jian et al., 2015) in a variety of human cancer cells including colorectal cancer cells. Nrf2 activation also can enhance the resistance of cancer cells to chemotherapeutic drugs.(Huang et al., 2015; Nakamura and Miyoshi, 2010) Therefore, the present results provide evidence that the combination with the PI3K inhibitor is a potential strategy to overcome resistance against food-derived anticancer compounds activating the Nrf2 pathway.

CONCLUSION

In the present study, the role of the PI3K/Akt/FoxO pathway in antiproliferation induced by BITC was investigated in human colorectal cancer cells. I also investigated the role of PI3K in BITC-related autophagy and its association with autophagy-related Nrf2/Keap1 pathway in human colorectal cancer cells.

In chapter 2, the role of the PI3K/Akt/FoxO pathway in antiproliferation induced by BITC in human colorectal cancer cells was investigated. The results are summarized as followed

(1) BITC exhibited anti-proliferative effect on human HCT-116 cells, whereas simultaneously activated PI3K/Akt/FoxO survival pathway in HCT-116, HT-29 and DLD-1 human colon cancer cells.

(2) Inhibition of PI3K/Akt pathway enhanced BITC-induced anti-proliferation in HCT-116, HT-29 and DLD-1 cells.

(3) MEK/ERK pathway was ruled out in the mechanism of BITC-induced drug resistance in human colon cancer cells.

(4) BITC significantly decreased PTP1B enzyme activity and enhanced sensitivity to insulin-induced PI3K/Akt/FoxO pathway as a PTP1B inhibitor.

The present results provide evidence that the combination with inhibitors of the PI3K/Akt/FoxO survival pathway is a promising therapeutic strategy to overcome resistance against food-derived anticancer compounds.

In chapter 3, I investigated the effect of BITC on autophagy in HCT-116 colon cancer cells and the role of PI3K in BITC-related autophagy and its association with autophagy-related Nrf2/Keap1 pathway. The results are summarized as followed.

(1) BITC enhanced the protein levels of representative molecules of autophagy, including p62, LC3BII and LAMP1 in a time-dependent manner up to 12 h.

(2) BITC activated the Nrf2 pathway in an autophagy-dependent manner (noncanonical pathway) in human colorectal cancer HCT-116 cancer cells.

(3) Inhibition of PI3K attenuated BITC-induced accumulation of autophagic molecules, accumulation of p62 and Nrf2 and HO-1 gene expression in human HCT-116 colon cancer cells.

The present results provide evidence that the combination with the PI3K inhibitor is a potential strategy to overcome resistance against food-derived anticancer compounds activating the Nrf2 pathway.

Taken together, these series of studies suggested that PI3K exceedingly contributed to drug-induced resistance not only in regulation of PI3K/Akt pathway but also in controlling in Nrf2/Keap1-related cellular detoxifying effects in human colorectal cancer cells. Combinative treatments with PI3K inhibitor are a potential strategy to overcome resistance against food-derived anticancer compound.

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