

Three bufadienolides-induced human lung cancer CL1-5 cell death mainly through autophagy

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Abstract

Lung cancer is almost the most common cause of cancer death in the world. Clinically, the conventional therapy to eradicate the cancer cells is chemotherapy but a better drug remains required. In this study, the effects of three bufadienolides, kalantuboside B, kalantuboside A and bryotoxin C, isolated from *Kalanchoe tubiflora* (Harvey) were evaluated and characterized in CL1-5 highly metastatic human lung cancer cells. Contrary to the apoptosis-promoting activity in other cancer cells, these three bufadienolides did not induce apoptosis in CL1-5 cancer cells. Instead, they activated an autophagy pathway, as indicated by the increase of autophagosomes formation. The induction of autophagy by these three bufadienolides was demonstrated to link to down-regulation of p-mTOR as well as up-regulation of LC3-II, ATG5, ATG7, Beclin-1. Moreover, among these three compounds, kalantuboside B in which a monosaccharide is attached at the bufadienolide aglycon, exhibited a better autophagy induction. Our findings revealed an alternative mechanism of drug action by bufadienolides in lung cancer cells and provided evidence for the possibility of treating highly metastatic human lung cancer through an autophagy pathway.

KEYWORDS: *Kalanchoe tubiflora*, bufadienolide, CL1-5 human lung cancer cells, autophagy

1. Introduction

Bufadienolides are characterized by the presence of C-24 steroid structure and a six-membered lactone (α -pyrone) ring located at C-17 β [1]. They have been found and isolated from plant and animal sources [1,2]. Pharmaceutical studies showed that bufadienolides possess potent anticancer activity. For example, bufalin has been demonstrated to induce apoptosis in gastric cancer MGC803 cells [3]. Bufalin induced apoptosis in human leukemia cells [4, 5]. Cinobufagin induced apoptosis in human breast cancer MCF-7 cell [6]. Bufalin was also demonstrated to increase expressions of autophagy markers, Beclin 1 and LC3II in hepatocellular carcinoma HepG2 cells [7]. Bufalin and cinobufagin induced apoptosis of HepG2 cells through both Fas- and mitochondria-mediated pathways [8]. Bufalin induced autophagy through Akt/mTOR pathway and apoptosis in liver cancer SK-HEP-1 cells [9]. *In vivo* examination of anti-human hepatocellular carcinoma in nude mice, bufalin exhibited a tumor inhibitory effect [10]. Bufalin induced apoptosis in human lung cancer NCI-H460 cells and exhibited the activity against human lung cancer xenografts [11]. Cinobufagin induced intrinsic apoptosis through AKT signaling pathway in human non-small cell lung cancer cells, including A549, H1299, H460, and SK-MES-1 [12]. In the clinical cases, 40% of patients suffer from a stage III or IV of non-small cell lung cancer, pancreatic cancer or hepatocellular carcinoma maintained a stable disease with the treatment of bufadienolides-containing toad venom [13]. Interestingly, bufalin only triggered autophagy in human colon cancer HT-29 and Caco-2 cells [14]. It is indicated that bufadienolides can induce apoptosis- or/and autophagy-mediated cell death in different cancer cells. However, the molecular pathways triggered by

bufadienolides seems various in different cancer cells.

Lung carcinoma is the most or second common cause of cancer-related deaths worldwide. In the clinic, although there are several therapeutic drugs such as Gefitinib and Erlotinib, an intrinsic or acquired resistance often occur [15, 16]. Thus, it is necessary to develop an effective chemotherapeutic drug to treat lung carcinoma. Moreover, no report elucidates the effect of bufadienolides on highly metastatic human non-small cell lung cancer cells, CL1-5. To discover the good candidate drug for the lung cancer therapy, in this present study, three bufadienolides were used to examine and compare their anticancer activities and explore the underlying mechanism of the cell death in the three bufadienolides-treated human lung cancer CL1-5 cells.

2. Materials and methods

2.1. Cell culture

CL1-5 highly metastatic human lung cancer cell line was kindly provided by Dr. C.C. Lin from National Chung Hsing University, Taiwan. Low-glucose Dulbecco's modified Eagle's medium with 1% penicillin-streptomycin and 10% FBS (Gibco Life Technologies, Grand Island, NY, USA) were used as growth medium. Cells were cultured at 37°C with 5% carbon dioxide. Cells at passages 4 to 16 were used for the examinations.

2.2. Bufadienolides

Three bufadienolides, kalantuboside B, kalantuboside A and bryotoxin C (Fig. 1), used in this study were isolated from *Kalanchoe tubiflora* (Harvey) as described previously [17].

2.3. Cell viability assay

CL1-5 cells were seeded into 24-well plates (10^4 cells each well) in 1 ml of growth medium and incubated overnight before drug treatments. Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay as described previously [18].

2.4. Annexin V/PI analysis

CL1-5 cells were seeded into 6-well plates. 0.05% trypsin-EDTA (Gibco, Gaithersburg, MD, USA) were used to detach the cell layer after drug treatments. The cell pellet was resuspended in PBS then stained with PI/Annexin V solution in the dark for 20 minutes according to the manual protocol (Invitrogen, Carlsbad, CA, USA). The treated cells were then analyzed by flow cytometry.

2.5. Cell cycle analysis

After drug treatments, CL1-5 cells were harvested and fixed with 70% methanol in PBS at 4°C overnight. Cells were washed twice to ensure removal of methanol before stained with PI and RNase.

Samples then put on flow cytometry after 30 minutes of PI staining.

2.6. Acridine orange staining analysis

CL1-5 cells were plated in 6-well for 24 hours before drug treatments. After drug treatments, acridine orange was added into culture medium with a working concentration of 10 µg/ml. Cells were incubated with acridine orange for 15 minutes and then analyzed by flow cytometry immediately.

2.7. LC3II puncta formation assay

LC-3II puncta formation assay was performed as described previously [18]. Briefly, CL1-5 cells were transfected with p-GFP-LC3 plasmids and then treated with vehicle or three compounds (100 nM) for 24 h, respectively. The green fluorescent LC3II puncta formation was observed under confocal microscopy.

2.8. Western blotting analysis

CL1-5 cells were plated in 6-cm dishes for 24 hours before drug treatments. After treatment with these three compounds, the harvested cells were lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was determined using a BCA kit (Thermo Fisher Scientific). Equal amounts of the proteins were separated by SDS-PAGE. The proteins were then electrotransferred to PVDF membranes (Millipore, Temecula, CA, USA) and detected by different

specific antibodies and secondary antibodies linked horseradish peroxidase. The membranes were developed using an enhanced chemiluminescence (ECL) system (Thermo Fisher). The detected protein bands were imaged by luminescence machine. The intensity of protein bands was quantified using Image J Software. The folds of the changes were calculated by dividing the intensity of the protein bands of the experimental group by those of control group.

3. Results

3.1. Kalantuboside B (KB), kalantuboside A (KA) and bryotoxin C (BC) reduce cell viability in human lung cancer CL1-5 cells

The CL1-5 human lung adenocarcinoma cells were used and the effects of KB, KA, BC treatment on CL1-5 cell viability were firstly examined with MTT assay. The results showed that the cell viabilities were inhibited by these three compounds in a dose-dependent manner. The IC₅₀ for 24 hr of KB, KA and BC treatment in CL1-5 cells was 52.30± 1.8 ng/ml (90.8 nM), 70.56± 5.7 ng/ml (114.18 nM) and 76.70± 1.3 ng/ml (162.49 nM), respectively (Fig. 2).

3.2. KB, KA and BC almost did not induce apoptosis

Apoptosis is a most common process of cell death. Thus, apoptosis induction by these three compounds was examined using Annexin V/PI analyses. Surprised, no significant apoptotic cells could be

detected (Fig. 3).

3.3. Only KB slightly induced G2/M cell cycle arrest

Cell cycle arrest is also a process of cell death. Cell cycle arrest by these three compounds was next examined. Interestingly, no significant cell cycle arrest could be observed in the KA- and BC- treated cells and only a slight increase of G2/M stage cells was observed in the KB-treated cells (Fig. 4). In addition, no obvious subG1 stage cell which represents apoptotic cell was detected. This result is consistent with that in the Annexin V/PI analyses. Since the slightly or no effect of three compounds on the cell cycle of the treated cells, it is expected that the contribution of the CL1-5 cell death would be another pathway.

3.4. KB, KA and BC increased autophagosomes

Autophagy process was next examined. CL1-5 cells were treated with KB, KA and BC for 24hrs, respectively, the cells were then stained with 1 µg/ml of acridine orange to observe the autophagosome caused by cellular autophagy using flow cytometry. Interestingly, the results showed that 14.45, 12.29 and 11.35% of cells exhibit greater autophagosomes after 100 nM of KB, KA and BC treatment, respectively (Fig. 5).

3.5. LC3 puncta formation in KB- and KA-treated CL1-5 cells

The increase of LC3 (microtubule-associated protein light chain 3) puncta is a promising indication for the induction of autophagosomes [19,20]. When autophagosomes are started to form, LC3-II will be increased and recruited to form the membrane of autophagic vesicles, results in the formation of punctate structures within the cytoplasm [20]. To confirm KB, KA and BC induce cellular autophagy, the LC3 aggregation was examined using overexpressing LC3-GFP fusion proteins in CL1-5. The plasmid contained with LC3-GFP was transfected into CL1-5 cells and the cells were treated with 100 nM of KB, KA and BC as well as 50 μ M of the positive control rapamycin for 24hrs, respectively. Using confocal microscopy, puncta structure formation was observed in the cells treated with KB, KA and the positive control rapamycin, but was not easy to be observed in the cells treated with BC (Fig. 6). Also, the puncta formation was much highly induced by KB than KA.

3.6. KB, KA and BC down-regulated p-mTOR and up-regulated LC3-II, Beclin-1, Atg5 and Atg7 in CL1-5 cells

Down-regulation of p-mTOR and up-regulation of LC3-II, Beclin-1, Atg5 and Atg7 have been shown to activate cellular autophagy [21, 22]. Thus, in order to elucidate the molecular effect, the levels of these proteins were examined by western blot analyses. As shown in Fig. 7, the level of p-mTOR was reduced and the levels of LC3-II, Beclin-1, Atg5 and Atg7 were increased by these three compounds and rapamycin. In addition, KB always exhibited higher activity.

3.7. KB, KA and AB decreased p62 in CL1-5 cells

p62 which is also named sequestosome 1 is a LC3-binding protein and a receptor for the cargo destined to be degraded by ubiquitinated proteins. As the autophagy-mediated degradation progresses, p62 is degraded by autophagy [23]. To elucidate this phenomenon, p62 was next detected. As shown in Fig. 8, the level of p62 were reduced in the CL1-5 cells treated with KB, KA, BC and rapamycin, confirming that these three compounds indeed induce cellular autophagy-mediated degradation process in the CL1-5 cells. In order to further confirm the autophagy induced by these three compounds, 3-methyladenine the early stage of autophagy inhibitor was used. The result showing the levels of p62 were reversed by the 3-methyladenine treatment in the cells treated with these three compounds confirmed that these three compounds indeed induce autophagy pathway in the CL1-5 cells. Interestingly, the level of p62 was not completely reversed in KB-treated cells. One possibility is the concentration of the inhibitor is not enough to block completely the induction of the autophagy induced by KB. Another possibility is that KB also induces a downstream signaling pathway beyond the inhibition of 3-methyladenine.

4. Discussion

In other cancer cells, cytotoxicity of KB, KA and BC were reported previously and their IC_{50} were showed at very low concentrations [17]. Moreover, the apoptotic cell death was shown to induce by KB,

KA and BC to HL-60 cells [17]. This study is the first time report that the cell viability of CL1-5 highly metastatic human lung cancer cells is remarkably decreased when treated with KB, KA or BC. The IC_{50} for 24 hr of KB, KA and BC treatment to CL1-5 cells are 90.8, 114.18 and 162.49 nM, respectively.

Numerous studies showed that apoptosis or a reciprocal action between apoptosis and autophagy is the major process to control cell fates [8, 9, 12, 24, 25]. Interestingly, the findings of this study showed that bufadienolides KB, KA, and BC do not induce cellular apoptosis but only induced cell autophagy.

The autophagy was confirmed by showing down-regulation of p-mTOR and up-regulation of LC3-II, Beclin-1, Atg5 and Atg7 in CL1-5 cells. In addition, the 3-methyladenine inhibitor was used to confirm the autophagy induction and also revealed that KB, KA, and BC act in the early stage of the autophagy process. In addition, KB also acts in the late stage of the autophagy process as 3-methyladenine inhibitor did not completely restore the content of p62, which is degraded in the very late stage of the autophagy. Our finding indicated for the first time that bufadienolides induce cell autophagy rather than apoptosis and KB exhibits the best induction activity in lung adenocarcinoma CL1-5 cells.

Comparing these three bufadienolide derivatives, BC contains a special 1,3,5-orthoacetate group, however, BC did not exhibit a better cytotoxic activity than KB and KA, suggesting that the 1,3,5-orthoacetate group is not an essential structure to perform the high cytotoxicity of the bufadienolide derivatives. This result is consisting of the previous finding of apoptosis induction in other cancer cells [17]. KB and KA containing a saccharide showed a higher cytotoxicity and autophagy induction, suggesting that a saccharide located at C-3 is able to increase its cytotoxic efficacy. This result is also

consisting with the previous finding of apoptosis induction in other cancer cells [17]. Moreover, a hydroxyl group rather than an acetoxy group located at C-3' exhibits a higher efficiency.

Autophagy was thought to be a cellular cytoprotective process to degrade damaged organelles and recycle proteins to against some stresses [26, 27, 28]. In some cases, autophagy and apoptosis modulate each other to control cell fates [24,25,29]. It has also been known that autophagy acts as a pro-death pathway and is called autophagic cell death which is different from apoptotic cell death [30, 31].

Interestingly, the findings in this study, the cell death caused by bufadienolides KB, KA, and BC almost did not induce cellular apoptosis but only induced cell autophagy. The result is similar to that a bufadienolide, bufalin, only triggers autophagy in human colon cancer HT-29 and Caco-2 cells [14]. As the close interplay between autophagy and apoptosis processes is crucial in the pathophysiology of non-small-cell lung cancer [29], KB, KA, and BC will be the potential drugs that can raise a synergetic effect with conventional anticancer drugs which mainly induce apoptosis pathway. This will be a very interesting issue to be demonstrated for the further study.

5. Conclusion

Kalantuboside B, kalantuboside A and bryotoxin C, isolated from *Kalanchoe tubiflora* (Harvey) activated autophagy process to induce cell death in CL1-5 highly metastatic human lung cancer cells. Among these three compounds, kalantuboside B exhibited a better autophagy induction and cytotoxicity.

This study provided evidence for the possibility of treating highly metastatic human lung cancer with bufadienolides.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Structures of the three bufadienolides used in this study.

Figure 2. Cell viability assays. CL1-5 cells were treated with different concentrations of kalantuboside B (KB), kalantuboside A (KA) and bryotoxin C (BC) for 24hr, and then assessed by the MTT assay. Data are presented the mean±S.D. of triplicate wells. The IC₅₀ values were calculated as 52.30 ± 1.8 ng/ml (90.8 nM), 70.56 ± 5.7 ng/ml (114.18 nM) and 76.70 ± 1.3 ng/ml (162.49 nM) for KB, KA and BC, respectively.

Figure 3. Annexin V-FITC and PI analyses. CL1-5 cells were treated with 100 nM of KB, KA and BC for 24 h, respectively. The horizontal and vertical axes represent labeling with Annexin V-FITC and Propidium Iodide-PE, respectively. The lower right part represents early apoptotic cells (positive for Annexin V only). The upper right part represents late apoptotic cells (positive for both Annexin V and PI).

Figure 4. Cell cycle analyses. CL1-5 cells were treated with KB, KA and BC (100 nM) for 24 hrs, respectively. Cell cycle distributions were determined by propidium iodide staining and subsequent flow cytometry analysis.

Figure 5. Autophagosomes measured by acridine orange staining. CL1-5 cells were treated 100 nM KA, KB and BC for 24 hrs, respectively, and then subjected to acridine orange staining and flow cytometry assays.

Figure 6. LC3-GFP puncta analyses in transfected CL1-5 lung adenocarcinoma cells. The CL1-5 cells transfected with LC3-GFP plasmid were treated with 100 nM of KB, KA and BC with 50 nM rapamycin in the CL1-5 cells for 24hr, respectively. The green fluorescence in the transfected cells was observed and detected by confocal microscopy.

Figure 7. The expression levels of autophagy-related proteins. Cells were treated with 50 nM rapamycin, 100 nM KB, KA and BC respectively. The antibody used for the western blot indicated at the right. The intensity of the band was quantitatively determined with ImageJ software. The ratios were determined by normalizing the intensity of the target bands with that of β -actin. Experiments were repeated three times with reproducible results.

Figure 8. The decreases of p62 were reversed by the inhibitor 3-MA. The CL1-5 cells were treated with 100 nM of KB, KA or BC, and with or without 5 mM of 3-MA for 24hr, respectively. The intensity of the band was quantitatively determined with ImageJ software. The ratios were determined by normalizing the intensity of the target bands with that of β -actin. Experiments were repeated three times with reproducible results.

Fig. 1

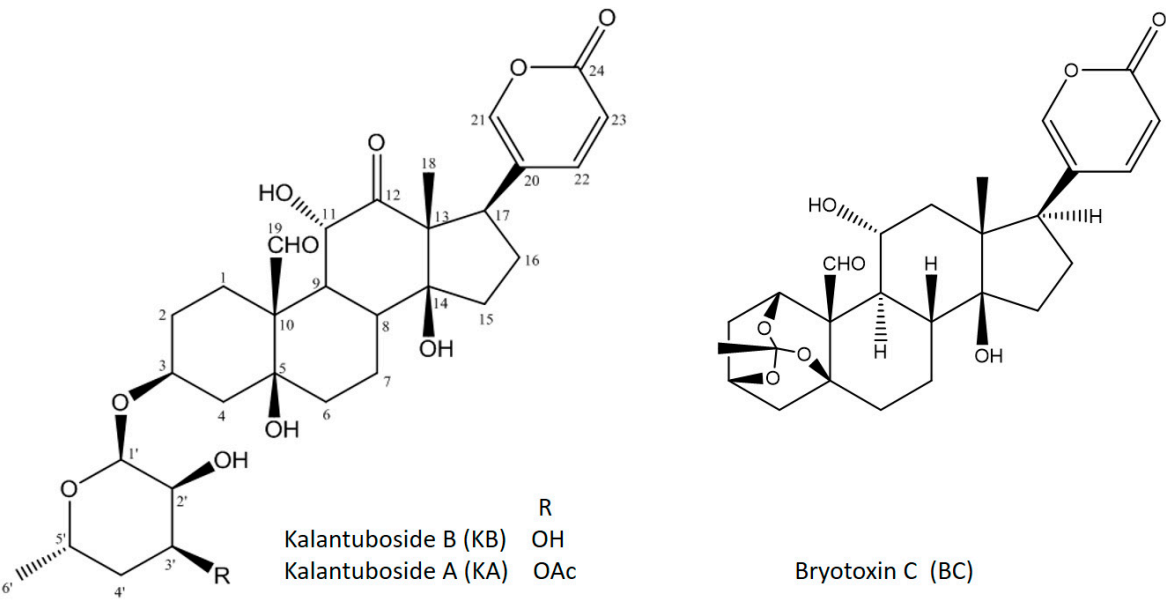


Fig. 2

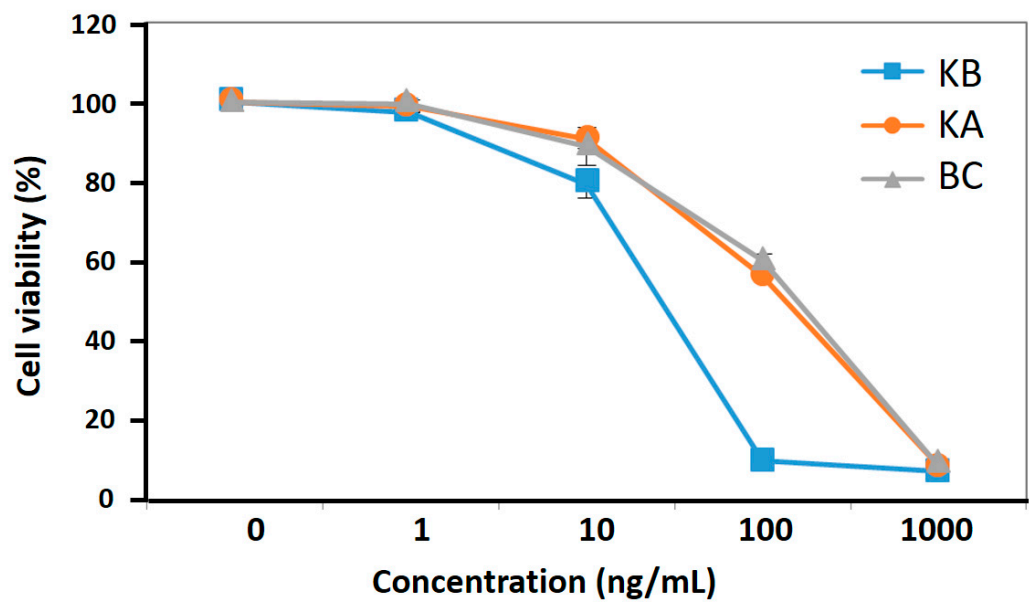


Fig. 3

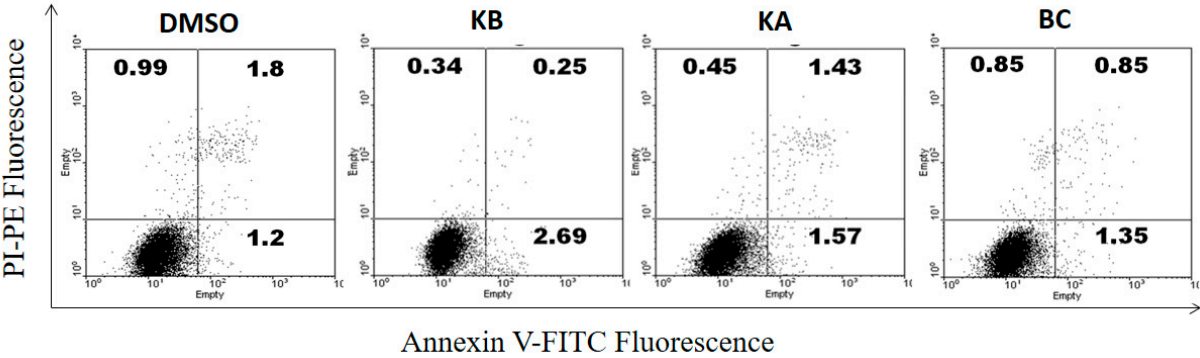


Fig. 4

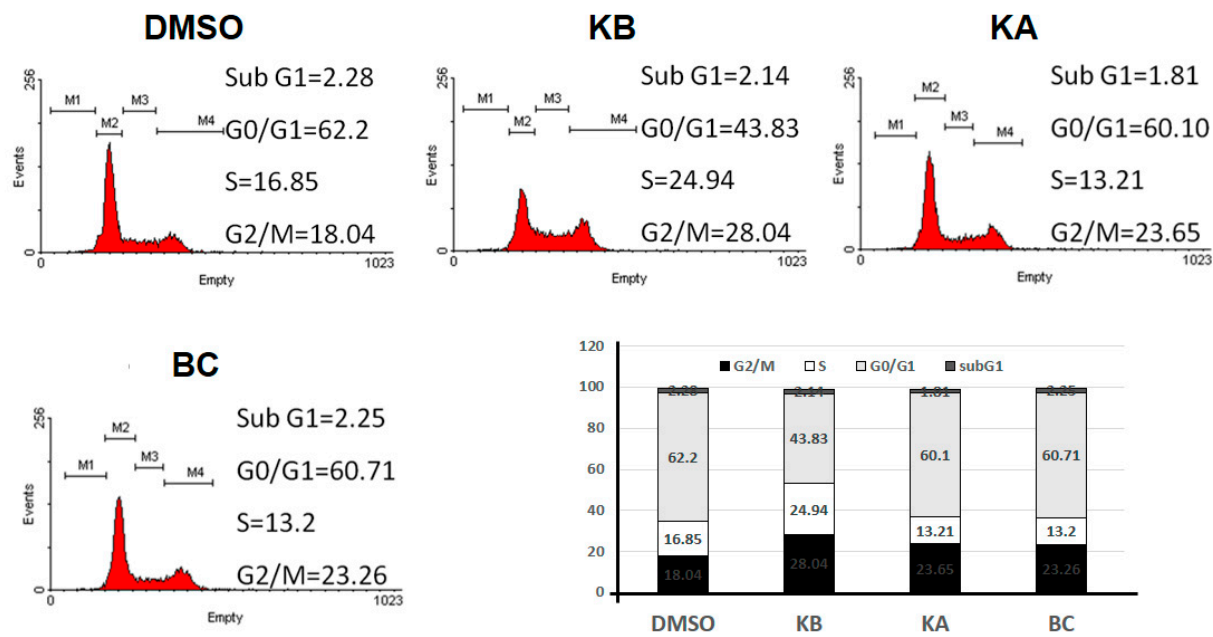


Fig. 5

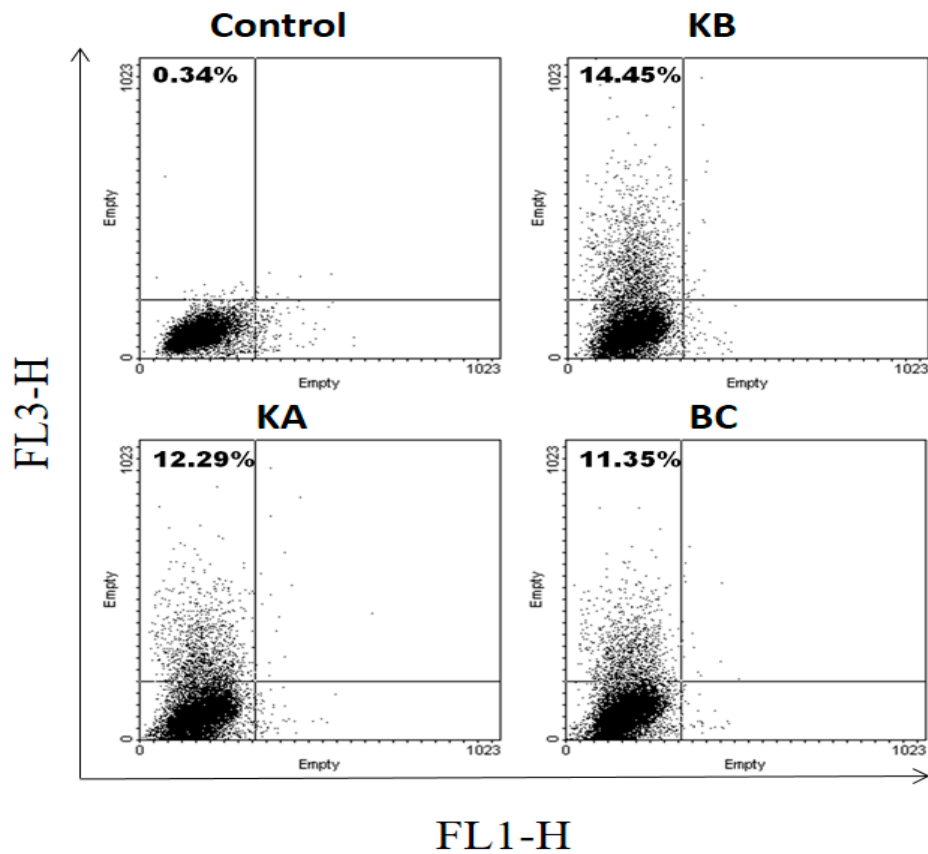


Fig. 6

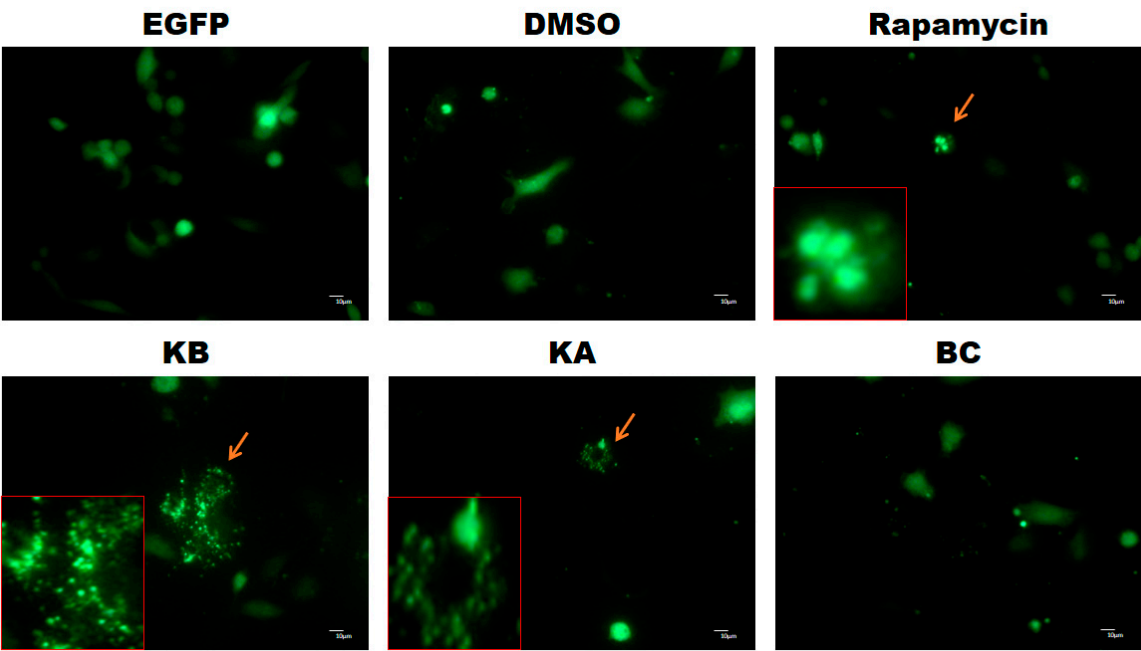


Fig. 7

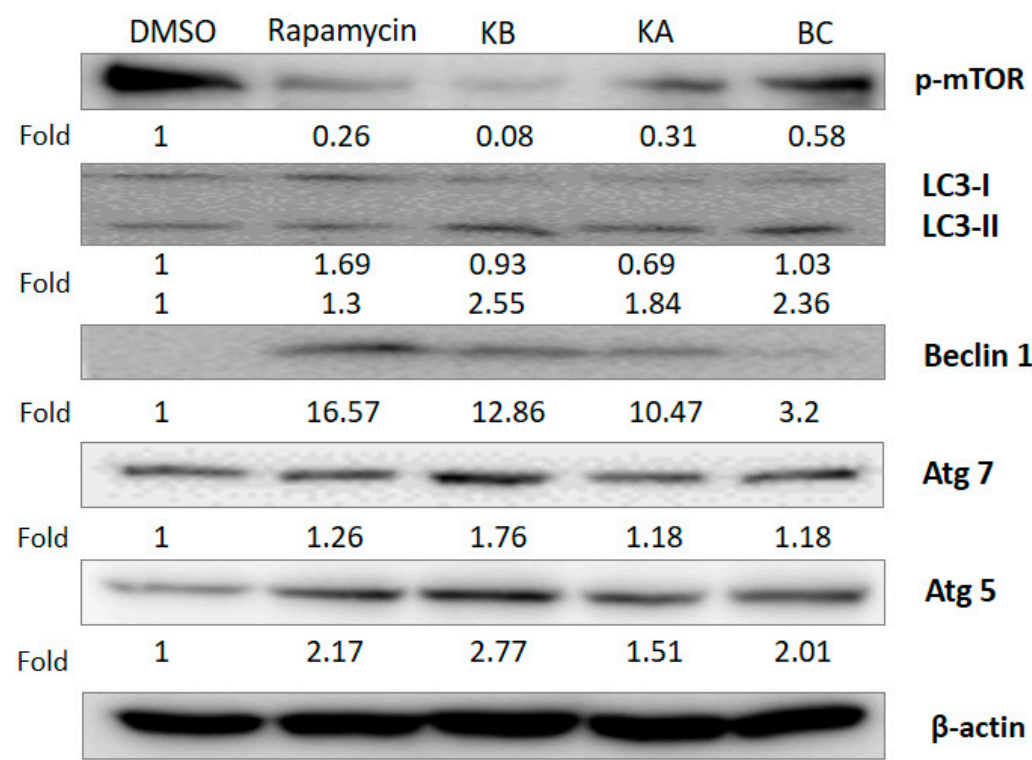


Fig. 8

