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Tetrandrine Increases the Sensitivity of Human Lung Adenocarcinoma PC14 Cells to Gefitinib by Lysosomal Inhibition

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Abstract. *Background/Aim: Human lung adenocarcinoma PC14 cells without mutations in the epidermal growth factor receptor (EGFR) are less sensitive to gefitinib than PC9 cells with EGFR mutations. We report the involvement of tetrandrine in autophagy flux as a mechanism that enhances the sensitivity of PC14 cells to gefitinib. Materials and Methods: Sensitivity to gefitinib was determined by a growth inhibition assay, and quantitative real-time PCR, western blotting, and fluorescent immunostaining were used to detect autophagy. Results: In PC14 cells, combined treatment with gefitinib and tetrandrine caused a significant increase in gefitinib sensitivity and autophagy-related mRNAs and proteins (LC3, etc.), and the LC3 protein accumulated in lysosomes. Furthermore, an autophagy flux assay revealed that tetrandrine inhibited lysosomes and that gefitinib promoted autophagy. Finally, the sensitivity of PC14 cells to gefitinib was enhanced with chloroquine. Conclusion: Tetrandrine possibly increases the susceptibility of PC14 cells to gefitinib by lysosomal inhibition.*

Lung cancers are the deadliest cancers in Japan, and the rate of adenocarcinoma is the highest of these cancers. Recently, it has been reported that the prevalence of *EGFR* gene mutations in Japanese lung adenocarcinoma is 45% (1). Treatment with the EGFR-tyrosine kinase inhibitor (EGFR-TKI) has been reported to be successful in gene mutation-positive patients (2, 3), and research is progressing with respect to the approval of molecule-targeted drugs (4, 5) for acquired resistance (6, 7). However, EGFR-TKI-insensitive lung adenocarcinoma accounts for approximately 50% of cases. Human lung adenocarcinoma PC14 cells that do not have an *EGFR* mutation are less sensitive to an EGFR-TKI, gefitinib, compared to human lung adenocarcinoma PC9 cells that carry an *EGFR* gene mutation (8).

Tetrandrine, a bisbenzylisoquinoline alkaloid isolated from *Stephania tetrandra*, has recently been reported to promote autophagy in several cells, including breast and liver cancer cells (9, 10). We found that tetrandrine enhances gefitinib sensitivity of PC14 cells. Here, using PC14 cells as an EGFR-TKI-insensitive lung adenocarcinoma model, we investigated the involvement of autophagy in the mechanism by which tetrandrine enhances gefitinib sensitivity.

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Key Words: Human lung adenocarcinoma PC14 cells, tetrandrine, gefitinib, autophagy flux, lysosomal inhibition.

Materials and Methods

Reagents. Tetrandrine was purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO, USA); gefitinib was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA); chloroquine diphosphate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); Fetal Bovine Serum (FBS) was purchased from Mediatech, Inc. (Corning, NY, USA); EDTA and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Dojindo Laboratories (Kumamoto, Japan); RPMI 1640 medium, Penicillin-Streptomycin-Amphotericin B Suspension (×100) (Antibiotic-Antimycotic Solution), dimethyl sulfoxide (DMSO), and other general reagents (special grade) were purchased from FujiFilm-Wako Pure Chemical Co., Inc. (Osaka, Japan); NucBlue™ Fixed Cell ReadyProbes™ Reagent (4',6-diamidino-2-phenylindole, DAPI) was purchased from Invitrogen (Eugene, OR, USA).

Cell culture. PC9 and PC14 cells were transferred from Dr. Toshiro Kasahara, Department of Cell Transplantation, Kanazawa University Hospital (currently Kanazawa University Hospital, Department of Respiratory Medicine). The cells were cultured in 100-mm dishes at a density of approximately $2.5\text{-}5.0\times10^5$ cells per dish in RPMI 1640 medium supplemented with 10% FBS at 37˚C under 95% air/5% CO_2. Drugs were added in the following order: 1) chloroquine, 2) tetrandrine, 3) gefitinib.

Gefitinib sensitivity test. Cells (2.0×10^3 cells/well) were plated in 96-well plates (100 μl/well) and incubated for 24 h at 37˚C. The

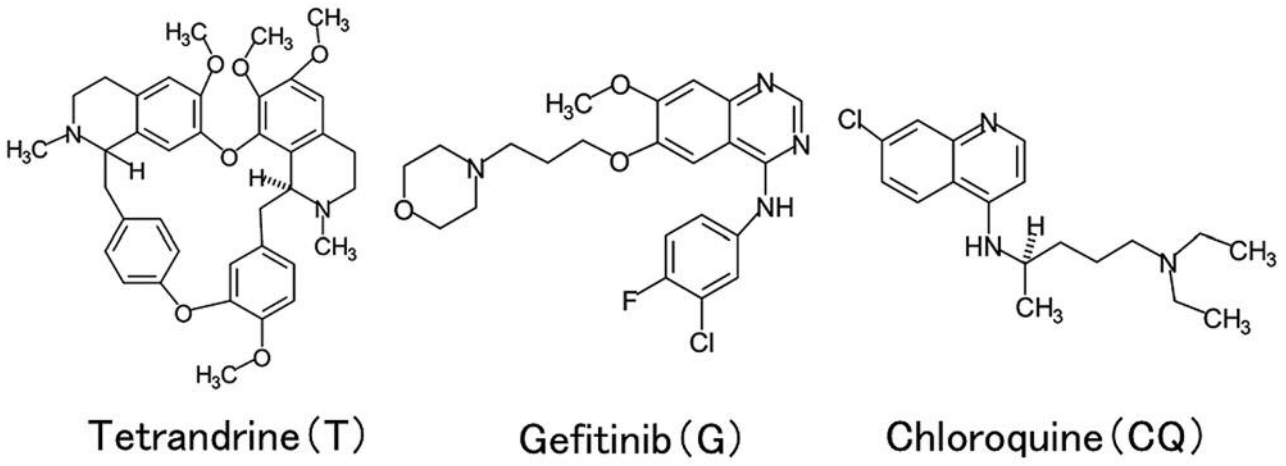

Figure 1. *Chemical structures of tetrandrine, gefitinib, and chloroquine.*

following day, 100 μl of medium containing the required drug concentration were added in each well. The cells were allowed to proliferate for 72 h at 37˚C in a humidified atmosphere of 5% CO_2. At the time of measurement, 20 μl of MTT reagent (5 mg/ml) were added to each well and incubated for 4 h. The generated formazan in cells was dissolved in DMSO, the absorbance was measured at 550 nm (A_{550}), and the value was obtained by subtracting the blank (medium alone) from the A_{550} values of the samples. The effect of drugs on cell growth was assessed as the percentage of cell viability.

Quantitative real-time PCR analysis. The mRNA expression levels of *Beclin1, ATG5, LC3A, LC3B, p62*, and *GAPDH* (as an internal control) in drug treated-PC14 cells (2×10^6) were determined by quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted using ISOGEN II® (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNAs were synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were carried out as previously reported (11). The primers were purchased from Life Technologies Japan Ltd. (Tokyo, Japan) and had the following sequences: for Beclin1, forward 5'-CTGGACACGAGTTTCAAGATCCT-3' and reverse 5'-TGTGGTAAGTAATGGAGCTGTGAGTT-3'; for ATG5, forward 5'-GCAGATGGACAGTTGCACACA-3' and reverse 5'-TTTCCCCATCTTCAGGATCAA-3'; for LC3A, forward 5'-TCCC GGACCATGTCAACAT-3' and reverse 5'-ACCATGCTGTGCTG GTTCAC-3'; for LC3B, forward 5'-ACCATGCCGTCGGAGAAG-3' and reverse 5'-ATCGTTCTATTATCACCGGGATTTT-3' ; for p62, forward 5'-AGGCGCACTACCGCGAT-3', and reverse 5'-CGTCACTGGAAAAGGCAACC-3'; for GAPDH, forward 5'-AAGCTCATTTCCTGGTATGACAACG-3', and reverse 5'-TCT TCCTCTTGTGCTCTTGCTGG-3'. Target gene expressions were calculated relative to GAPDH expression.

Western blot analysis. Cells (4×10^6) were lysed with triple detergent lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_2, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA•2Na, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin] and sonicated. Cell lysates were then collected by centrifugation at 15,000 ×*g* for 5 min at 4˚C. The protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Proteins (6.5-25 μg) were separated by 10-15% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). After blocking, the membrane was incubated overnight with primary antibodies against LC3 (1:2000, MBL, Nagoya, Japan), p62/SQSTM1 (1:1000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and β-actin (1:2000, Cell Signaling Technology Japan, K.K., Tokyo, Japan) followed by incubation with the corresponding HRP-conjugated secondary antibodies (Cell Signaling Technology Japan). Antibody-bound proteins were detected by ImmunoStar® Zeta (FujiFilm-Wako), and bands were analyzed using Fusion Solo 7S (M&S Instruments Inc., Osaka, Japan).

Immunofluorescence microscopy. Cells (2.5×10^3) were plated onto 8-well μ-slides (ibidi, GmbH., Gräfelfing, München, Germany). The following day, medium containing the required drug concentration was added to each well, and the cells were allowed to proliferate for 24 h (3 μM tetrandrine and/or 10 μM gefitinib) or 13 h (50 μM chloroquine) at 37˚C in a humidified atmosphere of 5% CO_2. After the treatment, the cells were fixed with cold methanol (–20˚C). The cells were washed once with phosphate buffered saline (PBS) and blocking was performed with 0.01% Triton X-100/ 3% BSA-PBS. Then, cells, except for the blank, were immunostained with the LC3 antibody (1: 4000, MBL, Nagoya, Japan) and the LAMP1 antibody (1: 2000, Santa Cruz Biotechnology) and incubated overnight at 4˚C. The cells were washed with PBS and incubated with two secondary antibodies (1:3000, anti-rabbit-Alexa Fluor® 488 and anti-mouse-Alexa Fluor® 555, Cell Signaling Technology Japan) in each well for 1 h at room temperature. The cells were washed with PBS and stained for 10 min with DAPI. After washing with PBS, the cells were added to the mounting medium and analyzed using a LSM510 confocal laser scanning microscope (Zeiss Japan, Tokyo, Japan).

Statistical analysis. All experiments were performed in duplicate, and the results were reported as means with standard deviations. The data were analyzed by an ANOVA Scheffe's multiple *t*-test.

Results

Tetrandrine enhances gefitinib sensitivity of PC14 cells. The chemical structures of the drugs used in this study are shown in Figure 1. PC14 cells are less sensitive to gefitinib than human lung adenocarcinoma PC9 cells that carry an EGFR gene mutation (8) (Figure 2A). The half maximal inhibitory concentration (IC_{50}) values were 0.02 μM and 19.7 μM for PC9 cells and PC14 cells, respectively. The cell growth of PC14 cells by treatment with 1 μM and 3 μM tetrandrine alone was 96.8±4.5% and 99.2±6.1%, respectively, of the untreated control. The concentration range of tetrandrine had no toxicity in PC14 cells. The combined treatment with tetrandrine increased the sensitivity of PC14 cells to gefitinib in a concentration-dependent manner, and there were almost no cells after treatment with 3 μM tetrandrine and 10 μM gefitinib for 72 h (Figure 2B). For this reason, subsequent experiments were conducted at this treatment concentration.

Combined treatment with tetrandrine and gefitinib in PC14 cells increases mRNA expression of autophagy-related proteins. In order to examine whether autophagy is involved in the gefitinib sensitivity enhancement effect of tetrandrine in PC14 cells, the mRNA levels of autophagy-related proteins were measured using quantitative real-time PCR. As a result, a significant increase in LC3A, LC3B, and p62 mRNA expression was observed by combined treatment with 3 μM tetrandrine and 10 μM gefitinib in PC14 cells for 24 h (Figure 3).

Combined treatment with tetrandrine and gefitinib in PC14 cells increases the expression of autophagy-related proteins. In response to the increase of mRNA expression levels of LC3 and p62 in PC14 cells treated with 3 μM tetrandrine and 10 μM gefitinib, the expression levels of proteins were quantified by western blotting (Figure 4A). The LC3 protein is widely used for autophagy monitoring, and the amount of LC3-II is known to correlate with the number of autophagosomes (12). The presence of LC3 in the autophagosome and the conversion to low mobility LC3-II with phosphatidylethanolamine added to LC3 was used as an indicator of autophagy (13), and the amount of autophagy was shown as LC3-II/LC3-I (14, 15). Cell lysate of PC14 cells treated with 50 μM chloroquine (CQ) for 13 h was used as a positive control for increasing LC3 protein. The LC3-II band increased with any treatment compared to the control, and the LC3-II/LC3-I ratio was also increased significantly by any treatment (Figures 4A and B). The ratios of LC3-II/LC3-I increased in the following order: control (C), 10 μM gefitinib (G), and 3 mM tetrandrine (T). In addition, the ratio in the combined treatment with 3 μM tetrandrine and 10 μM gefitinib (T+G) was much larger than that of the single

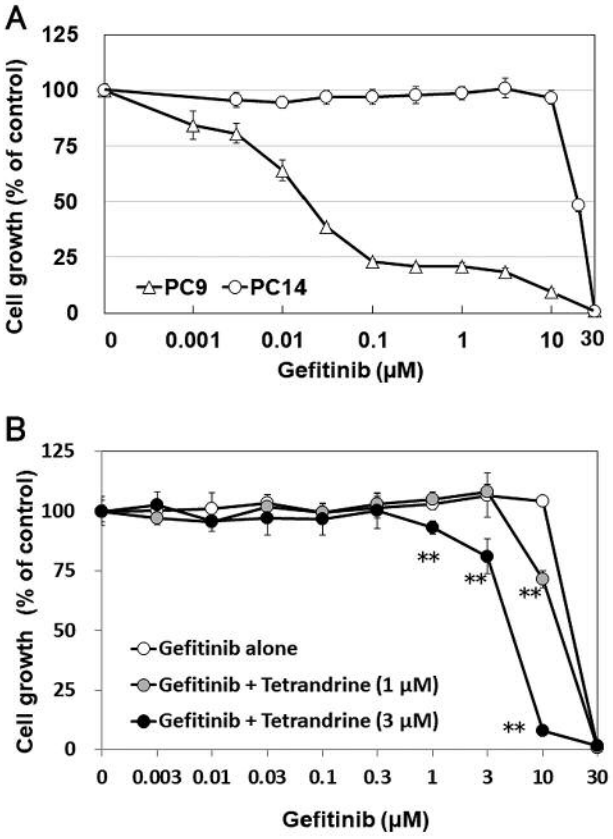

Figure 2. *Tetrandrine increases the gefitinib sensitivity of PC14 cells. (A) Effects of gefitinib on cell growth of PC9 and PC14 cells were measured by MTT assay. Human lung adenocarcinoma PC9 cells (2×10^3) and PC14 cells (2×10^3) were cultured with the indicated concentration of gefitinib for 72 h. Data are presented as the means±SD (n=7-8). (B) Effect of tetrandrine on the growth inhibition of gefitinib in PC14 cells was measured by MTT assay. Cells (2×10^3) were cultured with the indicated concentrations of gefitinib with tetrandrine (0, 1, 3 μM) for 72 h. Data are presented as the means±SD (n=4-6). **Significantly different from gefitinib alone at p<0.01.*

treatments, and it increased to the level of the LC3 protein positive control. In addition, the increase in the LC3-II/LC3-I ratio upon the combination treatment (T+G) was synergistic compared to tetrandrine alone and gefitinib alone.

A protein central to the process of constant autophagy, p62, is known to interact with various molecules including LC3 (16-18). Treatment with 3 μM tetrandrine (T), 10 μM gefitinib (G), combination of 3 μM tetrandrine and 10 μM gefitinib (T+G), and 50 μM chloroquine (CQ) caused a significant increase in p62 expression compared to the control (C) (Figures 4A and C), but the increase due to the combined treatment with tetrandrine and gefitinib (T+G) was additive, not synergistic.

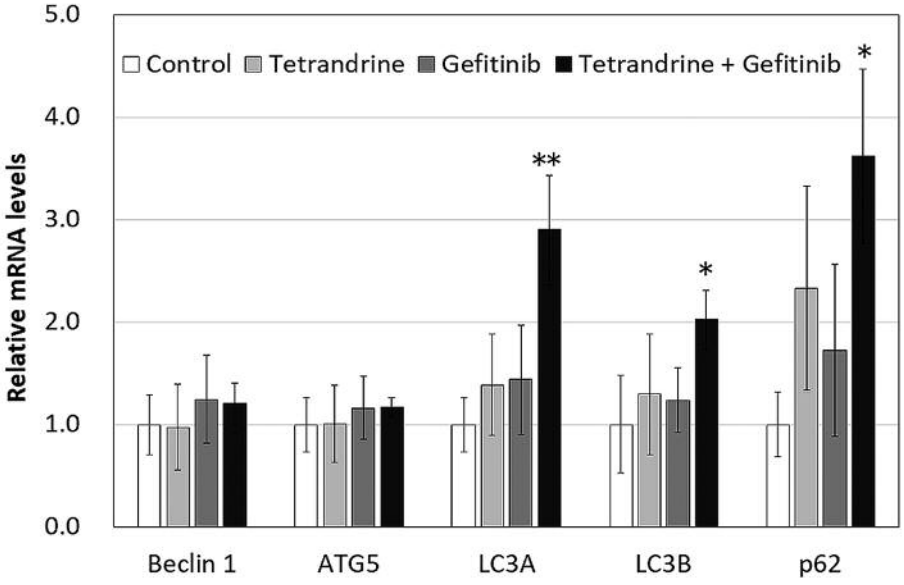

Figure 3. *Combined effects of tetrandrine and gefitinib on the mRNA expression of autophagy-related proteins. PC14 cells were treated with 3 μM tetrandrine and/or 10 μM gefitinib for 24 h. Beclin1, ATG5, LC3A, LC3B, and p62 were measured by quantitative real-time PCR. Target gene expression was calculated relative to GAPDH expression. *, **Significantly different from control at p<0.05 and p<0.01, respectively.*

Colocalization of the LC3 protein in lysosomes as a result of combined treatment with tetrandrine and gefitinib. The autophagosome marker protein LC3 and the lysosomal marker protein LAMP1 were fluorescently immunostained, and their images were photographed with a confocal microscope (Figure 5). LC3 (green) was almost absent in the control, showed a slight increase with 10 μM gefitinib treatment, and showed a marked increase in fluorescence with the treatments of 3 μM tetrandrine, combination of 3 μM tetrandrine and 10 μM gefitinib (Tet + GF), and 50 μM chloroquine. The result of LAMP1 staining was weak in the nucleus and in few lysosomes in the control and after treatment with 10 μM gefitinib; however, the treatments of 3 μM tetrandrine, Tet + GF, and 50 μM chloroquine showed a strong fluorescence intensity, indicating a biased LAMP1 accumulation in the cells. From the merged image of LC3 and LAMP1, LC3 showed almost no overlap with LAMP1 in the control and very little overlap in the treatment with gefitinib alone; however, the increased LC3 overlapped with LAMP1 in the treatment with tetrandrine alone (yellow). In the combined treatment with tetrandrine and gefitinib (Tet + GF), the lysosomes swelled and the LC3 signal was overpowered (orange). In the chloroquine treatment, approximately half of the increased LC3 overlapped with LAMP1.

Confirmation of autophagy induction by the autophagy flux assay. Although LC3-II is localized in the isolation and autophagosome membranes, the presence or absence of autophagy induction cannot be determined simply by an increase in the LC3-II band in western blotting. When a lysosomal inhibitor is added to a sample, autophagy induction is inferred if LC3-II expression increases; the treatment is assumed not to induce autophagy if the inhibitor has no effect (12). PC14 cells were treated with 3 μM tetrandrine and/or 10 μM gefitinib in the presence or absence of 10 μM chloroquine as a lysosomal inhibitor (19), and an increased LC3-II expression was confirmed with western blotting (Figure 6A). The increase in the LC3-II/LC3-I expression caused by the treatment of tetrandrine plus chloroquine was additive, but the increase caused by the treatment of gefitinib plus chloroquine was synergistic (Figure 6B).

Increase of the LC3-II protein by gefitinib treatment. From Figures 4, 6A, and 6B, we concluded that autophagy was possibly weakly induced by treatment with 10 μM gefitinib for 24 h. The increase in the LC3-II/LC3-I band caused by gefitinib was confirmed in a concentration-dependent manner (Figures 6C and D).

Enhancement of gefitinib sensitivity by chloroquine in PC14 cells. As shown in Figure 1B, tetrandrine enhanced gefitinib sensitivity in PC14 cells. It was confirmed that chloroquine exerted a similar effect when chloroquine was used in combination with gefitinib instead of tetrandrine. Combined treatment with chloroquine enhanced gefitinib sensitivity of PC14 cells in a concentration-dependent manner (Figure 7). The growth of PC14 cells after treatment with 10 μM and 20

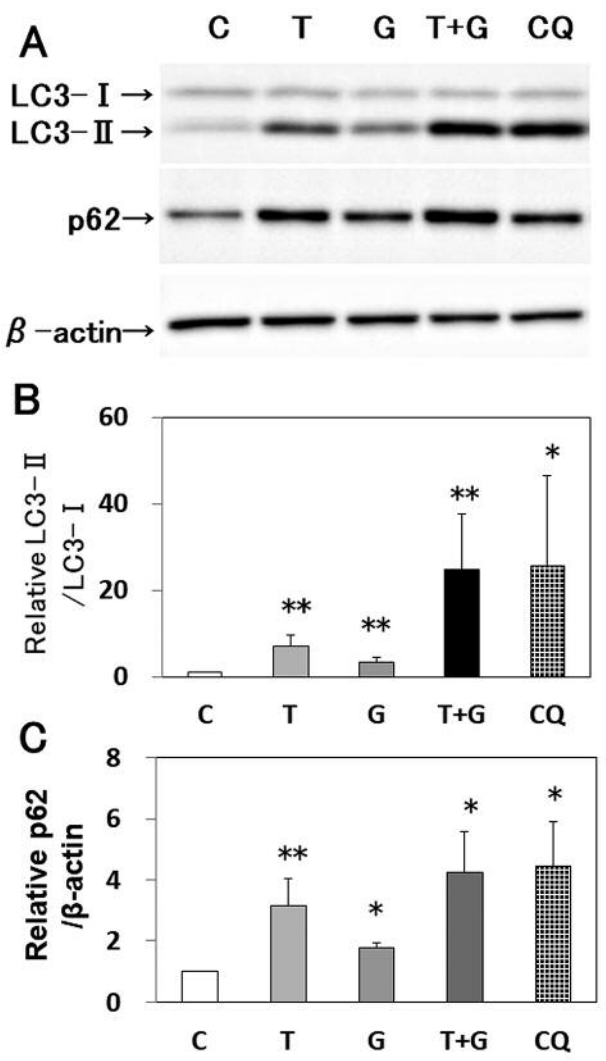

Figure 4. *Combined treatment with tetrandrine and gefitinib in PC14 cells increases the expression of autophagy-related proteins. Western blotting of LC3, p62, and β-actin proteins (A), relative LC3-II/LC3-I (B) and p62/β-actin (C) in PC14 cells treated with 3 μM tetrandrine and/or 10 μM gefitinib for 24 h, or 50 μM chloroquine for 13 h. C: Control, T: 3 μM tetrandrine, G: 10 μM gefitinib, T+G: combined treatment with 3 μM tetrandrine and 10 μM gefitinib, CQ: 50 μM chloroquine. Data are presented as the means±SD (n= 4-5). *, **Significantly different from control at p<0.05 and p<0.01, respectively.*

μM chloroquine alone was 99.1±3.6% and 97.4±3.1%, respectively, of the untreated control.

Discussion

PC14 cells were approximately 800 times less sensitive to gefitinib than human lung adenocarcinoma PC9 cells containing *EGFR* gene mutations (Figure 2A). When PC14 cells were treated with tetrandrine and gefitinib, marked cell growth inhibition was observed (Figure 2B). The purpose of this study was to elucidate the growth inhibition mechanism in EGFR-TKI-insensitive tumor cells.

Recently, a connection between autophagy and tetrandrine has been reported (9, 10). In this study, mRNA expression of autophagy-related proteins was measured by quantitative real-time PCR. Our results indicated that there were no changes in Beclin1 or ATG5 expression; however, a significant increase in p62, LC3A, and LC3B expression was observed with the combined treatment of tetrandrine and gefitinib (Figure 3). Macroautophagy consists of two processes: first, autophagosomes are formed by the extension of an isolation membrane to surround cytoplasmic components; second, the autophagosomes fuse with lysosomes, which results in the digestion of the components. The reason that tetrandrine did not increase the mRNA levels of *Beclin1* or *ATG5*, which are important factors in the first process of macroautophagy (20), but increased the mRNA levels of the *p62* and *LC3* proteins, which are involved in the second process of macroautophagy, is unknown. Lysosomal inhibition by tetrandrine may inhibit the protein degradation of p62 and LC3 based on another study that found a decrease in the intracellular amino acid pool and an upregulation in p62 and LC3 mRNA levels (21).

In our study, the LC3 protein was detected by western blotting, and its expression upon the TG combined treatment (T+G) was found to be synergistic when compared to tetrandrine alone (T) or gefitinib alone (G); however, the expression of p62 in TG combined treatment was found only to be additive (Figures 4A, B and C). Since it was not known whether the increase in LC3 expression was the result of increased autophagy or the result of lysosomal inhibition, an autophagy flux assay was performed in the presence and absence of chloroquine, a lysosomal inhibitor (Figure 6). The increase rate of the LC3-II/LC3-I expression by tetrandrine plus chloroquine treatment was additive, but gefitinib plus chloroquine treatment showed a synergistic increase (Figures 6A and B). In addition, an increase in the LC3-II/LC3-I expression levels by gefitinib treatment was confirmed (Figures 6C and D), suggesting an increase in autophagy flux with gefitinib treatment. Considering our results and the interpretation of the LC3 immunoblotting (12), we hypothesized that tetrandrine inhibits lysosomes and that gefitinib causes autophagy.

In addition, the p62 protein levels increase in response to autophagy inhibition and the inhibition of autophagy flux (21, 22, 23). However, it is known that an increase in autophagy flux also increases p62 protein levels (21, 24, 25). Considering the results shown in Figure 6, the increase in p62 expression may be due to the inhibition of autophagy flux by tetrandrine and the increase in autophagy flux by gefitinib. Unlike the synergistic relationship represented by the LC3 band, it is unclear why the p62 band intensity of the TG combination treatment (T + G) remained additive with

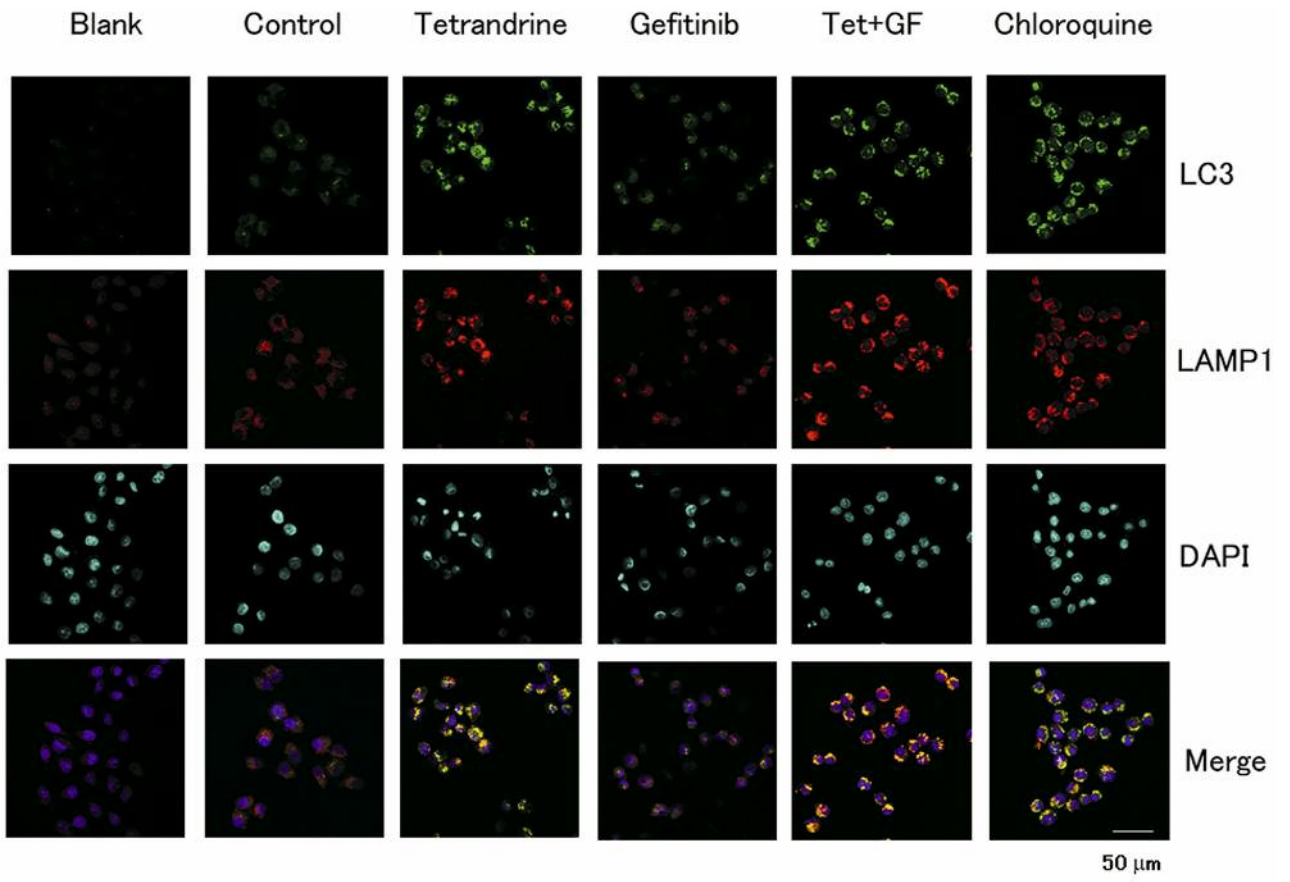

Figure 5. *Confirmation of colocalization of the LC3 protein to lysosomes using fluorescent immunostaining by confocal microscopy. PC14 cells were treated with 3 μM tetrandrine and/or 10 μM gefitinib for 24 h, or 50 μM chloroquine for 13 h. The autophagosomal marker protein, LC3 (green), and lysosomal marker protein, LAMP1 (red), were fluorescently immunostained, and the nucleus was stained with DAPI (blue). Tet + GF indicates combined treatment with tetrandrine and gefitinib. Bar, 50 μm.*

respect to the tetrandrine alone (T) and gefitinib alone (G) treatments. A clear correlation between LC3-II and p62 is not always found, and when they are transcriptionally upregulated, the interpretation of the results is more complex (21). Therefore, further investigation is needed.

Furthermore, much information was obtained in the confirmation experiment of the intracellular localization of the LC3 protein (green) and lysosomal marker protein LAMP1 (red) by fluorescent immunostaining (Figure 5). In particular, the stained image of the combined treatment with tetrandrine and gefitinib indicated that lysosomal inhibition was caused by tetrandrine during degradation of the cell contents after autolysosome formation. This is based on the fact that Bafilomycin A1, a V-type ATPase-specific lysosomal inhibitor, does not localize with LC3 in the lysosome and remains green and red when merged; it does not turn yellow (26). In contrast, treatment with chloroquine resulted in an image that was half green and half yellow (Figure 5, chloroquine). In addition, other studies have reported that chloroquine blocks fusion with lysosomes and prevents autolysosome clearance and cargo content degradation, but does not prevent autophagosome or lysosome fusion (27-30). It has also been hypothesized that the mechanism could be specific to each cell type (21).

Chloroquine, at least partially, inhibits the fusion of autophagosomes and lysosomes; in addition, it also suppresses the degradation of the cell contents in autolysosomes that have not been inhibited and have advanced to the second process. We believe that this explains why half of the image was green and half was yellow (Figure 5, chloroquine). The results of the combined treatment with tetrandrine and gefitinib (Tet+GF) showed that the lysosomes swelled and that the green color of LC3 was overpowered, resulting in a color that was almost orange (Figure 5,

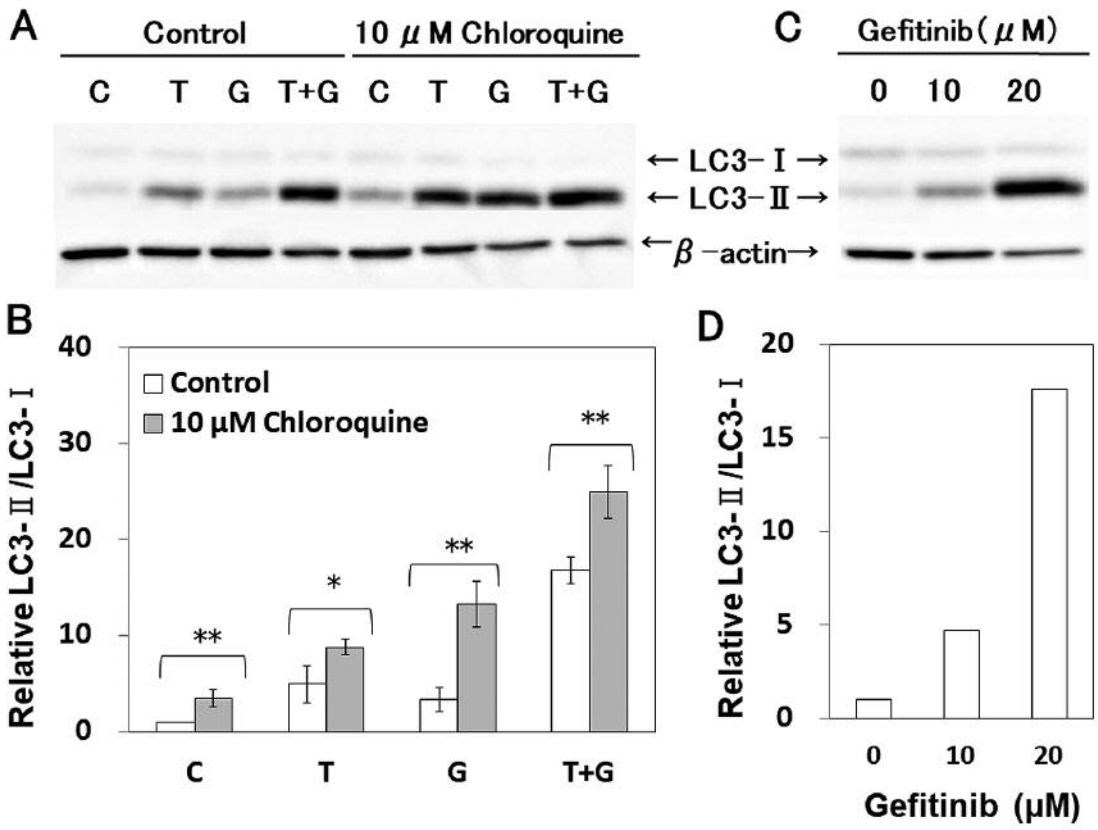

Figure 6. *Confirmation of autophagy induction by the autophagy flux assay. Western blotting of LC3 proteins (A) and relative LC3-II/LC3-I (B) in PC14 cells treated with tetrandrine and/or gefitinib in the presence or absence of 10 μM chloroquine for 24 h. C: Control. T: 3 μM tetrandrine. G: 10 μM gefitinib. T+G: combined treatment with 3 μM tetrandrine and 10 μM gefitinib. Drugs were added in the following order: 1) chloroquine, 2) tetrandrine, 3) gefitinib, and the interval between each treatment was 30 min. Data are presented as the means±SD (n= 3). *, **Significantly different from control (without chloroquine) at p<0.05 and p<0.01, respectively. Western blotting of LC3 and β-actin proteins (C) and relative LC3-II/LC3-I (D) in PC14 cells treated with gefitinib (0, 10, 20 μM) for 24 h.*

Tet+GF). Based on the results of comparative studies with chloroquine in our experiments, tetrandrine likely does not inhibit the pre-fusion process of autophagosomes and lysosomes, but may act as a lysosomal inhibitor that strongly suppresses post-fusion degradation in PC14 cells.

As tetrandrine has been suggested to inhibit lysosomes, we hypothesized that chloroquine, a lysosome inhibitor, would increase the sensitivity of PC14 cells to gefitinib.

Chloroquine enhanced gefitinib sensitivity (Figure 7), suggesting that lysosome inhibition is a mechanism of enhancement of gefitinib sensitivity by tetrandrine.

In conclusion, tetrandrine may block gefitinib-induced autophagy flow and enhance gefitinib sensitivity in PC14 cells by lysosomal inhibition. Because autophagy provides a great source of energy and materials for cancer cell growth, its inhibition can lead to cancer cell growth arrest. We hope that this result will help the design of cancer treatment strategies in the future.

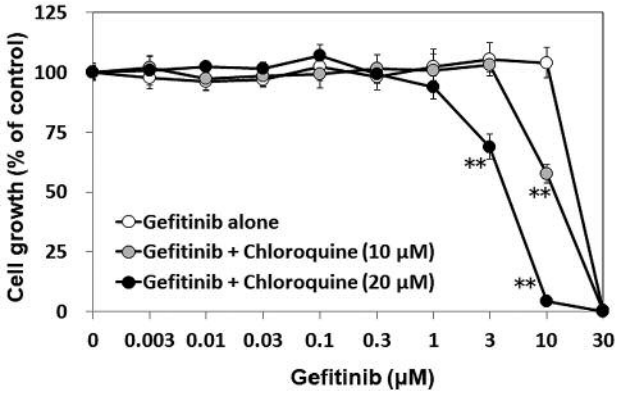

Figure 7. *Chloroquine enhances the gefitinib sensitivity of PC14 cells. Effects of chloroquine on growth inhibition of gefitinib in PC14 cells were measured by MTT assay. PC14 cells (2×10^3) were cultured with the indicated concentrations of gefitinib and chloroquine (0, 10, 20 μM) for 72 h. Data are presented as the means±SD (n=3-6). **Significantly different from gefitinib alone at p<0.01.*

Conflicts of Interest

The Authors declare no conflicts of interest associated with this manuscript.

Authors' Contributions

Masaaki Nomura designed the study, and assisted in the preparation of the manuscript. Eiko Sato wrote the initial draft of the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All Authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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References

1 Midha A, Dearden S and McCormack R: EGFR mutation incidence in non-small-cell lung cancer of adenocarcinoma histology: a systematic review and global map by ethnicity (mutMapII). Am J Cancer Res *5(9)*: 2892-2911, 2015. PMID: 26609494.

2 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350(21)*: 2129-2139, 2004. PMID: 15118073. DOI: 10.1056/NEJMoa040938

3 Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304(5676)*: 1497-1500, 2004. PMID: 15118125. DOI: 10.1126/science.1099314

4 Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, Orme JP, Finlay MR, Ward RA, Mellor MJ, Hughes G, Rahi A, Jacobs VN, Red Brewer M, Ichihara E, Sun J, Jin H, Ballard P, Al-Kadhimi K, Rowlinson R, Klinowska T, Richmond GH, Cantarini M, Kim DW, Ranson MR and Pao W: AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. Cancer Discov *4(9)*: 1046-1061, 2014. PMID: 24893891. DOI: 10.1158/2159-8290.CD-14-0337

5 Jänne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, Ahn MJ, Kim SW, Su WC, Horn L, Haggstrom D, Felip E, Kim JH, Frewer P, Cantarini M, Brown KH, Dickinson PA, Ghiorghiu S and Ranson M: AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med *372(18)*: 1689-1699, 2015. PMID: 25923549. DOI: 10.1056/NEJMoa1411817

6 Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG and Halmos B: EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med *352(8)*: 786-792, 2005. PMID: 15728811. DOI: 10.1056/NEJMoa044238

7 Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med *2(3)*: e73, 2005. PMID: 15737014. DOI: 10.1371/journal.pmed.0020073

8 Noro R, Gemma A, Kosaihira S, Kokubo Y, Chen M, Seike M, Kataoka K, Matsuda K, Okano T, Minegishi Y, Yoshimura A and Kudoh S: Gefitinib (IRESSA) sensitive lung cancer cell lines show phosphorylation of Akt without ligand stimulation. BMC Cancer *6*: 277, 2006. PMID: 17150102. DOI: 10.1186/1471-2407-6-277

9 Wong VKW, Zeng W, Chen J, Yao XJ, Leung ELH, Wang QQ, Chiu P, Ko BCB and Law BYK: Tetrandrine, an activator of autophagy, induces autophagic cell death *via* PKC-α inhibition and mTOR-dependent mechanisms. Front Pharmacol *8*: 351, 2017. PMID: 28642707. DOI: 10.3389/fphar.2017.00351

10 Mei L, Chen Y, Wang Z, Wang J, Wan J, Yu C, Liu X and Li W: Synergistic anti-tumour effects of tetrandrine and chloroquine combination therapy in human cancer: a potential antagonistic role for p21. Br J Pharmacol *172(9)*: 2232-2245, 2015. PMID: 25521075. DOI: 10.1111/bph.13045

11 Kobayashi S, Kawasaki Y, Takahashi T, Maeno H and Nomura M: Mechanisms for the anti-obesity actions of bofutsushosan in high-fat diet-fed obese mice. Chin Med *12*: 8, 2017. PMID: 28360931. DOI: 10.1186/s13020-017-0129-x

12 Mizushima N and Yoshimori T: How to interpret LC3 immunoblotting. Autophagy *3(6)*: 542-545, 2007. PMID: 17611390. DOI: 10.4161/auto.4600

13 Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y and Yoshimori T: LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci *117(Pt 13)*: 2805-2812, 2004. PMID: 15169837. DOI: 10.1242/jcs.01131

14 Bai W, Bai J, Li Y, Tian D and Shi R: Microtubule-associated protein 1S-related autophagy inhibits apoptosis of intestinal epithelial cells *via* Wnt/β-catenin signaling in Crohn's disease. Biochem Biophys Res Commun *485(3)*: 635-642, 2017. PMID: 28188784. DOI: 10.1016/j.bbrc.2017.02.034

15 Yang F, Liao J, Pei R, Yu W, Han Q, Li Y, Guo J, Hu L, Pan J and Tang Z: Autophagy attenuates copper-induced mitochondrial dysfunction by regulating oxidative stress in chicken hepatocytes. Chemosphere *204*: 36-43, 2018. PMID: 29649662. DOI: 10.1016/j.chemosphere.2018.03.192

16 Sánchez-Martín P, Saito T and Komatsu M: p62/SQSTM1: 'Jack of all trades' in health and cancer. FEBS J *286(1)*: 8-23, 2019. PMID: 30499183. DOI: 10.1111/febs.14712

17 Gatica D, Lahiri V and Klionsky DJ: Cargo recognition and degradation by selective autophagy. Nat Cell Biol *20(3)*: 233-242, 2018. PMID: 29476151. DOI: 10.1038/s41556-018-0037-z

18 Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, Kominami E, Yamane T, Tanaka K and Komatsu M: Structural basis for sorting mechanism of p62 in selective

autophagy. J Biol Chem *283(33)*: 22847-22857, 2008. PMID: 18524774. DOI: 10.1074/jbc.M802182200

19 de Duve C, de Barsy T, Poole B, Trouet A, Tulkens P and Van Hoof F: Commentary. Lysosomotropic agents. Biochem Pharmacol *23(18)*: 2495-2531, 1974. PMID: 4606365. DOI: 10.1016/0006-2952(74)90174-9

20 Nakatogawa H, Suzuki K, Kamada Y and Ohsumi Y: Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol *10(7)*: 458-467, 2009. PMID: 19491929. DOI: 10.1038/nrm2708

21 Klionsky DJ *et al*: Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy *12(1)*: 1-222, 2016. PMID: 26799652. DOI: 10.1080/15548627.2015.1100356

22 Jung J, Seo J, Kim J and Kim JH: Ursolic Acid Causes Cell Death in PC-12 Cells by Inducing Apoptosis and Impairing Autophagy. Anticancer Res *38(2)*: 847-853, 2018. PMID: 29374711. DOI: 10.21873/anticanres.12293

23 Komatsu M, Wang QJ, Holstein GR, Friedrich VL, Jr., Iwata J, Kominami E, Chait BT, Tanaka K and Yue Z: Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci USA *104(36)*: 14489-14494, 2007. PMID: 17726112. DOI: 10.1073/pnas.0701311104

24 Trocoli A, Bensadoun P, Richard E, Labrunie G, Merhi F, Schlafli AM, Brigger D, Souquere S, Pierron G, Pasquet JM, Soubeyran P, Reiffers J, Ségal-Bendirdjian E, Tschan MP and Djavaheri-Mergny MZ: p62/SQSTM1 upregulation constitutes a survival mechanism that occurs during granulocytic differentiation of acute myeloid leukemia cells. Cell Death Differ *21(12)*: 1852-1861, 2014. PMID: 25034783. DOI: 10.1038/cdd.2014.102

25 Toepfer N, Childress C, Parikh A, Rukstalis D and Yang W: Atorvastatin induces autophagy in prostate cancer PC3 cells through activation of LC3 transcription. Cancer Biol Ther *12(8)*: 691-699, 2011. PMID: 21768780. DOI: 10.4161/cbt.12.8.15978

26 Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J *19(21)*: 5720-5728, 2000. PMID: 11060023. DOI: 10.1093/emboj/19.21.5720

27 Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A and Thompson CB: Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest *117(2)*: 326-336, 2007. PMID: 17235397. DOI: 10.1172/JCI28833

28 Garcia-Garcia A, Anandhan A, Burns M, Chen H, Zhou Y and Franco R: Impairment of Atg5-dependent autophagic flux promotes paraquat-and MPP(C)-induced apoptosis but not rotenone or 6-hydroxydopamine toxicity. Toxicol Sci *136(1)*: 166-82, 2013. PMID: 23997112. DOI: 10.1093/toxsci/kft188

29 Maclean KH, Dorsey FC, Cleveland JL and Kastan MB: Targeting lysosomal degradation induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis. J Clin Invest *118(1)*: 79-88, 2008. PMID: 18097482. DOI: 10.1172/JCI33700

30 Poole B and Ohkuma S: Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J Cell Biol *90(3)*: 665-669, 1981. PMID: 6169733. DOI: 10.1083/jcb.90.3.665

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