Re-evaluation of the role of autophagy in thyroid cancer treatment

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Abstract. Numerous studies have examined the role of autophagy in thyroid cancer treatment; however there are discrepancies among the reported data, with some showing the pro-survival and others the anti-survival effects of autophagy. These discrepant results appear to be at least in part due to insufficient analyses or data misinterpretation as well as improper assessments of autophagic activity. Therefore, the present study re-evaluated the regulation of autophagic activity by various anticancer modalities and examined the role of autophagy in thyroid cancer treatment in three thyroid cancer cell lines (TPC1, ACT1 and KTC1). The immunofluorescence and DalGreen findings demonstrated that cisplatin, irradiation and sorafenib were all autophagy inducers as previously reported, but, unlike previous studies using thyroid cancer cells, doxorubicin acted as an inhibitor. KTC1 cells are unique because they only responded to cisplatin. The efficacy of anticancer therapeutics was significantly higher in chloroquine or 3-methyladenine-treated autophagy-defective cells than in autophagy-competent cells, thereby indicating the pro-survival effect of autophagy induced by anticancer therapeutics, which is partly due to inhibition of apoptosis. Thus, the present findings relating to several anticancer therapeutics and three thyroid cancer cell lines demonstrate the pro-survival effect of autophagy in thyroid cancer treatment. Although the present study only involved cell lines, it provides evidence for the beneficial combination of the anticancer therapeutic modalities with autophagy inhibitors, and proposes that autophagy inhibitors may serve as a possible adjunctive therapy for thyroid cancer.

Key words: Thyroid, Autophagy, Anticancer therapeutics, Radiation

THYROID CANCER is the most common endocrine malignancy, and its incidence has been increasing markedly over the last several decades [1]. Although differentiated thyroid cancer (including papillary thyroid carcinoma (PTC), and follicular thyroid carcinoma) has a good prognosis with a long-term survival rate of nearly 90\%, de-differentiated thyroid cancer [poorly differentiated and anaplastic thyroid carcinoma (ATC)] has a survival rate of less than 10\% [2]. Unfortunately, this number has remained unchanged for many years due to the limited treatment options for the latter group of patients who intrinsically have or develop a loss of response to conventional treatments such as surgery, radioactive iodine treatment and thyrrotropin suppression therapy in addition to cytotoxic chemotherapeutic drugs [3].

An extensive number of genetic and genomic studies have shown that point mutations of BRAF and RAS genes and chromosomal rearrangements of the RET receptor (RET/PTC) are the major driver mutations in PTC [4]. These findings have led to the introduction of tyrosine kinase inhibitors, such as sorafenib and vemurafenib, as a new therapeutic choice [5, 6]; however some patients show intrinsic resistance or develop acquired resistance to these targeted therapies. Thus, a novel therapeutic approach is urgently required.

Macroautophagy (herein referred to as autophagy) is an evolutionary process that catabolizes intracellular proteins and organelles to prevent their aging and provides energy when cells are in stressed conditions, such as starvation. It is generally accepted that autophagy has dual effects on cell survival and death. On one hand it supports cell survival by providing nutrients required for supporting cellular proliferation and survival, and maintaining homeostasis, but on the other hand, it promotes cell death through a mechanism known as autophagy-regulated cell death, including excessive self-digestion, excessive mitophagy and autosis [7]. Our recent study...
has also shown that autophagy plays an important role in survival and homeostasis of normal thyroid glands [8].

With regards to its physiological role, autophagy acts as a double-edged sword in cancer. Thus, it is generally considered that autophagy suppresses tumor development in the early stage of tumorigenesis, but it promotes tumor cell survival and proliferation once a tumor develops. Therefore, the majority of preclinical and clinical studies on cancer treatment have focused mainly on autophagy inhibition, but accumulated data have demonstrated that both enhancement and inhibition of autophagy have a therapeutic effect. This is also the case for thyroid cancer, where some studies have shown the pro-survival effect, whereas others have shown the anti-survival effects of autophagy on thyroid cancers [9]. For example, vemurafenib (a BRAF inhibitor), sorafenib (a pan-tyrosine kinase inhibitor), apatinib (a VEGF-2 inhibitor) and cisplatin (a chemotherapeutic drug) suppress cell survival and proliferation, and simultaneously induce autophagy. Moreover, the anticancer effects of these agents can be augmented by autophagy inhibitors including chloroquine (CQ), 3-methyladenine (3-MA) and small interfering (si) RNA for ATG5 or BECLIN-1 [10-16], indicating the pro-survival effect of autophagy on thyroid cancer cells. In contrast, the anticancer effects of doxorubicin, apigenin and allicin, all of which are reported autophagy inducers, were inhibited by autophagy inhibition [17-19], suggesting the anti-survival effect of autophagy. More complicating matters are inconsistent data from a number of other different studies. For example, autophagy inhibitors such as CQ, 3-MA or siRNA for components of autophagic machinery alone suppressed cell proliferation in some studies [12, 20-22], but this result was not found in others [13, 19, 23]. Moreover, a recent study demonstrated CQ-induced promotion of tumorigenesis [24]. The cytotoxic effect of radiation was enhanced in a human thyroid squamous cell cancer cell line SW-579 [25], but was diminished in TPC1 and 8505C cell lines [26], by autophagy inhibition. Autophagy inhibitors aggravated the anti-cancer effect of CZ415 (an mTOR1/2 inhibitor) in TPC1 cells [23] but abolished action of RAD001 (an mTOR1 inhibitor) in TPC1 and 8505C cells [26].

These discrepant results appear to be, at least in part, due to insufficient analyses, data misinterpretation and/or improper assessments of autophagic activity. Therefore, the present study re-evaluated the regulation of autophagic activity by various anticancer therapeutics and the role of autophagy in thyroid cancer treatment using thyroid cancer cell lines. Additionally, mechanistic analysis of cell death induced by anticancer therapeutics and autophagy inhibitors was performed.

Materials and Methods

Cell cultures

Three thyroid cancer cell lines with different driver mutations were used. TPC1 cells with RET-PTC chromosome rearrangement and TERT<sup>228C>T</sup> were obtained from Prof. J. Fagin (Memorial Sloan-Kettering Cancer Center, USA) (https://www.expasy.org/celllosaurus/CVCL_6298). KTC1 cells with BRAF<sup>V600E</sup> and TERT<sup>250C>T</sup> were kindly provided by Prof. J. Kurebayashi (Kawasaki Medical School, Kawasaki) (CVCL_6300). ACT1 cells with NRAS<sup>Q61K</sup>, TERT<sup>250C>T</sup> and TP53<sup>C242S</sup> (CVCL_6291) were obtained from Prof. N. Onoda (Osaka City University, Osaka; originally established by Dr. S. Ohata at Tokushima University [27]). Although the former two cell lines are from PTC, and the latter from ATC, most thyroid cancer cell lines are generally reported to be in a de-differentiated state after long-term in vitro culture [28, 29]. The cells were grown in RPMI1640 medium (TPC1 cells) or DMEM (KTC1 and ACT1 cells) (both from Sigma-Aldrich, St Louis, MD, USA) supplemented with 5% or 10% FBS, respectively, and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The absence of any mycoplasma infection was confirmed with the Venor GeM One Step mycoplasma detection kit (Minerva BioLabs, GmbH, Germany).

Treatment with anticancer agents

Sorafenib (cat. no. SRP0702-10MG; Sigma) stock solution was made at a concentration of 10 mM in DMSO and aliquots were kept at -20°C. Rapamycin (cat. no. tlr-ral; InvivoGen) was made at a concentration of 10 mM in DEMSO. CQ (cat. no. 08660-04, Nacalai Tesque, Inc) was made at a concentration of 10 mM in PBS. 3-MA (cat. no. sc-205596; Santa Cruz Biotechnology, Inc) was made at a concentration of 33.5 mM in culture medium. Doxorubicin (cat. no. X90030; Nippon Kayaku) was made at a concentration of 2.5 mg/mL in PBS. Cisplatin (cat. no. LAH0914; FUJIFILM Wako Pure Chemical Corporation) was made at a concentration of 10 mM in DMF.

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc) assay. The cells were trypsinized and seeded in a 96-well plate at a density of approximately 1 × 10<sup>4</sup> cells/mL in 200 μL, and on the following day they were incubated with various concentrations of chemicals or exposed to irradiation (see the “X-ray irradiation” section below). After 48 h, the cells were incubated in 50 μL of medium with 10% CCK-8 solution at 37°C for 1 h. The dye absorbance was
was defined as (mean treated – blank)/(mean untreated control – blank) × 100. Each group had ≥5 duplicated wells. The data are presented as means ± S.D.

**Clonogenic assay**

The cells were plated in 10-cm dish (1 × 10^5 cells/well), treated for 1 h with CQ or 3-MA and then exposed to irradiation. After 7 days, the cells were fixed for 10 min and stained with 1% crystal violet (FUJIFILM Wako Pure Chemical Corporation) in 10% ethanol for 10 min. The number of colonies containing ≥50 cells was counted. Graphs were normalized to 100% per treatment, and data are presented as means ± S.D. of three independent experiments performed in triplicates.

**X-ray irradiation**

X-ray irradiation was administered as a single dose of 2 or 10 Gy (15 kV, 200 mA; ISOVOLT Titan 320; GE Sensing & Inspection Technologies, Tokyo) at a fixed dose rate of 0.8903 Gy/min and a 50-cm focus distance.

**Immunofluorescence analysis**

The cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton-X. The primary and secondary antibodies used were: i) guinea pig polyclonal anti-p62 (cat. no. GP62-C, Progen; 1:100) and Alexa Fluor 488-conjugated goat polyclonal anti-guinea pig IgG (cat. no. ab150185, Abcam; 1:200) for p62; and ii) rabbit polyclonal anti-LC3 (cat. no. PM036, Medical & Biological Laboratories Co., Ltd; 1:1,000) and Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG (cat. no. A-11008; Thermo Fisher Scientific, Inc; 1:200) for LC3. The stained cell samples were analyzed using an All-in-One BZ9000 Fluorescence microscope (Keyence). In total, 100 cells were evaluated in each sample to determine the percentages of TUNEL-positive cells using Image J software.

**Evaluation of apoptosis**

The TUNEL method (determination of DNA fragmentation in apoptotic cells using TdT-mediated dUTP-digoxigenin nick end-labeling) was performed with the Apop-tag™ Fluorescein Direct in situ apoptosis detection kit (Sigma). The cells were fixed with 3.7% formaldehyde at 4°C for 25 min, permeabilized with 0.2% Triton-X for 5 min at room temperature and treated with 0.5 μg/mL proteinase K for 3 min. The cells were incubated with the equilibrium buffer at room temperature for ≥10 sec and with TdT enzyme in a humidified chamber at 37°C for 1 h. The reaction was terminated with stop/wash buffer for 10 min and an anti-digoxigenin conjugate was added for 30 min at room temperature. The cell samples were analyzed using an All-in-One BZ-9000 Fluorescence microscope (Keyence). In total, 1,000 cells were evaluated in each sample to determine the percentages of TUNEL-positive cells using Image J software.

**Statistical analysis**

The normality of the sample distribution was verified using the Shapiro-Wilk test. When the sample distribution was normal, the significant differences between the parameters of the two compared groups were determined using the two-tailed Student’s t-test. A p value <0.05 was considered to indicate a significant difference. Multiple comparison analysis was performed using one-way ANOVA followed by Tukey’s post hoc test. All data are presented as means ± SD.

**Results**

**Evaluation of autophagic flux in three thyroid cancer cell lines**

Whether autophagy functions normally in three thyroid cancer cell lines, TPC1, ACT1 and KTC1, was evaluated using a well-known autophagy inducer (rapamycin) and inhibitors (CQ and 3-MA). Autophagic activity was monitored by examining the number of LC3 puncta and p62 expression level, as described previously [9]. The data regarding TPC1 cells are shown in the main Figs., and those regarding ACT1 and KTC1 cells are provided in the Supplementary Figs. The dose-dependent cytotoxic assay (Fig. 1A) and the dose-dependent quantified at a wavelength of 450 nm (0.1 sec) using a PerkinElmer 2030 Multilabel Reader ARVO™ X3 (Perkin Elmer, Inc., Branchburg, NJ, USA). Response was defined as (mean treated – blank)/(mean untreated control – blank) × 100. Each group had ≥5 duplicated wells. The data are presented as means ± S.D. of three independent experiments performed in triplicates.

**Clonogenic assay**

The cells were plated in 10-cm dish (1 × 10^5 cells/well), treated for 1 h with CQ or 3-MA and then exposed to irradiation. After 7 days, the cells were fixed for 10 min and stained with 1% crystal violet (FUJIFILM Wako Pure Chemical Corporation) in 10% ethanol for 10 min. The number of colonies containing ≥50 cells was counted. Graphs were normalized to 100% per treatment, and data are presented as means ± S.D. of three independent experiments performed in triplicates.

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**Autophagy detection with DALGreen staining**

The cells seeded in a 96-well plate were incubated at 37°C for 30 min with 200 μL of 1 μM DALGreen working solution (Dojindo Molecular Laboratories, Inc), following which they were incubated with amino acid-free medium (FUJIFILM Wako Pure Chemical Corporation) or regular medium containing sorafenib, cisplatin or doxorubicin, or were exposed to irradiation. Then the cells were observed with an LSM-880 confocal laser microscope (Carl Zeiss AG) for up to 48 h. The fluorescence intensity was quantified using Image J and expressed as corrected total cell fluorescence (CTCF) relative to the control. CTCF = integrated density – (area of selected cell × mean fluorescence of background readings). For each condition, 50 cells were analyzed.
autophagic activity assay (data not shown) were performed using TPC1 cells, and the data relating to the autophagic activity obtained with the concentrations that induced cell death in up to approximately 20% of the cells (250 nM rapamycin, 1 mM 3-MA and 50 μM CQ) are presented in Fig. 1B and C. Rapamycin increased the number of LC3 puncta and decreased p62 expression, whereas 3-MA decreased LC3 puncta and increased p62 expression, and CQ increased both LC3 puncta and p62 expression. 3-MA and CQ inhibit autophagy at the early and late, respectively, stages of autophagic flux. Similar data were obtained from ACT1 and KTC1 cells (Figs. S1, S2A and B). These findings confirmed the enhancement of autophagy by rapamycin and its inhibition by 3-MA and CQ, indicating that the autophagic machinery in all three cell lines was functionally intact.

Fig. 1 Effects of Rap, 3-MA, CQ, Sor, Cis, Dox and irradiation (IR) on cell viability, p62 levels and LC3 puncta in TPC1 cells. (A) Cell viability assay. The cells were incubated with graded doses of chemicals or exposed to IR. After 48 h, cell viability was determined using a Cell Counting Kit-8 assay. (B and C) Immunofluorescence analyses of the number of LC3 puncta and p62 expression levels. The cells were incubated for 48 h with 250 nM Rap, 1 mM 3-MA, 50 μM CQ, 2 μM Cis, 0.2 μg/mL Dox or 100 nM Sor, or exposed to 10 Gy IR. Quantitative analyses of the number of LC3 puncta and p62 expression levels were performed. Original image magnifications, ×1,000 for LC3 and ×400 for p62. Data are presented as means ± SD (n = 3–5). *, p < 0.05 vs. Cont. Rap, rapamycin; 3-MA, 3-methyladenine; CQ, chloroquine; Sor, sorafenib; Cis, cisplatin; Dox, doxorubicin; IR, irradiation; Cont, control.
Regulation of autophagic flux by anticancer therapeutics in thyroid cancer cell lines

As mentioned earlier, due to the discrepant data reported on the role of autophagy on cancer cell treatment using mTOR inhibitors, irradiation and chemotherapeutic drugs (cisplatin vs. doxorubicin), the present study monitored autophagic flux in cells treated with these anticancer therapeutics. Sorafenib was also used as a positive control as an autophagy-inducing anticancer agent [12]. From the dose-dependent cytotoxic (Fig. 1A) and autophagic activity (data not shown) assay results in TPC1 cells, the data obtained with the concentrations that induced cell death in up to approximately 20% of the cells (100 nM sorafenib, 2 μM cisplatin, 0.2 μg/mL doxorubicin and 10 Gy irradiation) are presented in Fig. 1B and C. Sorafenib increased LC3 puncta and decreased p62 expression, confirming autophagy-induction by sorafenib. In contrast, irradiation and the anticancer drugs cisplatin and doxorubicin increased both LC3 puncta and p62 expression. These data suggest that irradiation, cisplatin and doxorubicin could be either an autophagy inhibitor (at the late step of autophagic flux similar to CQ) or an autophagy inducer, since p62 expression is occasionally increased at transcriptional/post-transcriptional levels and independently from autophagy [9]. To discriminate these two possibilities, the cells were co-exposed to irradiation or anticancer drugs and CQ. Again, sorafenib was used as a positive control. As presented in Fig. 2, further increases in LC3 puncta were observed in the cells treated with the combination of CQ with cisplatin, irradiation or sorafenib, but not doxorubicin, as compared with the cells treated with cisplatin, irradiation, sorafenib or CQ alone. These data indicated that cisplatin and irradiation (and sorafenib) are autophagy-inducers, whereas doxorubicin is an autophagy inhibitor. Similar results were also observed in ACT1 cells (Figs. S1 and S3).

However, in KTC1 cells (Fig. S2B), cisplatin notably increased LC3 puncta and decreased p62 expression, indicating induction of autophagy by cisplatin. Moreover, doxorubicin increased both LC3 puncta and p62 expression, and its combination with CQ showed no additive effect (Fig. S4), thereby confirming doxorubicin-inhibition of autophagy. However, no alterations in LC3 puncta and p62 expression were detected in the cells treated with irradiation or sorafenib, even in the dose-dependent experiments (Fig. S2C). Thus KTC1 cells are unique, in that they are responsive to cisplatin and doxorubicin, but unresponsive to irradiation or sorafenib; however, the mechanism remains unknown.

The regulation of autophagic flux by the above noted anticancer therapeutics was also examined with live imaging using DALGreen. DALGreen is an innovative fluorescent molecule that is incorporated into the autophagosomal membrane when the membrane is formed, and fluorescence intensity is enhanced under acidic pH conditions when an autophagosome fuses with a lysosome to form the autolysosome. This makes DALGreen suitable for monitoring the last step of autophagic flux [30]. Amino acid-free culture medium was used as a
positive control. In TPC1 cells in amino acid-free medium, increased DALGreen fluorescence intensity was first detected at 2 h and this reached a plateau at 24 h (Fig. 3). Sorafenib, cisplatin and irradiation similarly increased DALGreen fluorescence with different kinetics with the first significant increases in DALGreen fluorescence being observed at 6 h with sorafenib and cisplatin and at 24 h with irradiation, but doxorubicin showed no effect on DALGreen fluorescence. These data are consistent with the findings obtained regarding LC3 puncta and p62 (Figs. 1 and 2), thereby confirming that sorafenib, cisplatin and irradiation are autophagy inducers and that doxorubicin is an autophagy inhibitor. The same results were obtained for all the therapeutics in ACT1 cells (Fig. S5), and no alteration in DALGreen fluorescence was detected by sorafenib and irradiation in KTC1 cells (Fig. S6), which was consistent with the data shown in Figs. S1, S2 and S3. All of the above findings are summarized in Table 1.

**Effects of autophagy inhibition on the cytotoxic effects of autophagy inducers in thyroid cancer cell lines**

Given that rapamycin, sorafenib, cisplatin and irradiation are autophagy inducers, the subsequent step was to determine whether the autophagy induced by these therapeutics was pro- or anti-survival. For this purpose, their cytotoxic effects were compared between control (autophagy-competent) cells and CQ or 3-MA-treated (autophagy-defective) cells using cytotoxic and clonogenic assays. As shown in Fig. 4A, rapamycin, sorafenib, cisplatin and irradiation administered alone induced cell death in 0–26% of the cells in the cytotoxic assay in control, autophagy-competent TPC1 cells, which was significantly enhanced by up to 17–36% in CQ/3-MA-treated, autophagy-defective TPC1 cells. Moreover, in the clonogenic assay (Fig. 4B), 2 Gy of irradiation did not produce any noticeable decrease in the number of colonies in autophagy-competent cells, but did induce a significant decrease in colony numbers in autophagy-defective cells. These data demonstrate the pro-survival effect of autophagy induced by all the anticancer therapeutics examined.

The same results were obtained with ACT1 cells (Fig. S7). However, with KTC1 cells, although autophagy induced by rapamycin and cisplatin was clearly shown to be pro-survival, the cytotoxic effects of sorafenib and irradiation were not augmented in autophagy-defective cells compared with autophagy-competent cells (Fig. S8); data comparable with the above results show the inability of sorafenib and irradiation to induce autophagy (Figs. S2 and S6). Thus, the augmentation of the cytotoxicity of anticancer therapeutics by autophagy inhibitors, CQ and 3-MA, was only observed under the condition where anticancer therapeutics could induce autophagy.

**Effect of autophagy inhibition on apoptosis induction by autophagy inducers in TPC1 cells**

Finally, a TUNEL assay was performed to determine whether apoptosis was induced by the autophagy inducers, and, if so, how the degree of apoptosis was altered by autophagy inhibition. As shown in Fig. 5A and B, etoposide, a positive control for apoptosis induction, induced apoptosis in approximately 50% of TPC1 cells.
All of the autophagy inducers, rapamycin, sorafenib, irradiation and cisplatin, and the autophagy inhibitor CQ induced apoptosis (7.8–33.3%). A combination of these autophagy inducers with CQ induced significantly higher levels of apoptosis (40.7–85.6%) as compared with treatment with single agents. These data suggest that the pro-survival effect of autophagy in TPC1 cells is, at least in part, mediated by the suppression of apoptosis.

**Discussion**

As noted previously, numerous studies on the role of autophagy in thyroid cancer treatment have been conducted, some of which however reported discrepant data [9]. Therefore, the present study first re-evaluated the regulation of autophagic flux by various anticancer therapeutics using three thyroid cancer cell lines, and found that sorafenib, irradiation and cisplatin were autophagy inducers as previously reported [12, 15, 16, 25, 31]. Unlike the findings from a previous report [31], however, doxorubicin acted as an inhibitor. Although other studies have also reported doxorubicin-induction of autophagy, this misinterpretation appears to be due to insufficient analyses of autophagic flux, as only the amounts of LC3-II (or the number of LC3 puncta) and formation of autolysosomes, not p62 expression or formation of autolysosome, were determined [31-34].

| Cell lines (mutations) | Rapamycin | 3-methyladenine | Chloroquine | Sorafenib | Cisplatin | Irradiation | Doxorubicin |
|------------------------|-----------|----------------|-------------|-----------|-----------|-------------|-------------|
| TPC1 (RET/PTC)         | ↑         | ↓              | ↓           | ↑         | ↑         | ↑           | ↓           |
| ACT1 (NRAS<sup>Q61K</sup>) | ↑         | ↓              | ↓           | ↑         | ↑         | ↑           | ↓           |
| KTC1 (BRAF<sup>V600E</sup>) | ↑         | ↓              | ↓           | →         | ↑         | →           | ↓           |
Indeed, recent reports have shown that doxorubicin blocks autophagy at the late stage of autophagic flux by impairing lysosomal acidification, so that it increases both LC3-II and p62, which is similar to action of CQ [35-37]. These reports are consistent with the present data showing increased LC3 punctate formation and p62 expression, and decreased autolysosome formation, as detected via immunofluorescence and DALGGreen in doxorubicin-treated cells. Thus, doxorubicin likely inhibits autophagic flux and disrupts the autophagy process before the formation of autolysosomes. Similarly, the present study suggested insufficient analyses and improper interpretations of autophagic flux in some other studies on sanguinarine, alopeine and mulberry...
anthocyanin, all of which were shown to have an anti-survival effect of autophagy [18, 38, 39].

Notably, of three thyroid cancer cell lines examined in the present study, KTC1 cells, which harbor BRAF\(^{V600E}\), are unique in terms of their lack of autophagy response to sorafenib and irradiation despite their normal autophagy function (shown by their normal response to rapamycin, 3-MA, CQ and cisplatin). These data indicate that the effect of anticancer therapeutics on autophagic activity may be cell-context dependent. It is unlikely that this unique phenotype is attributed to BRAF\(^{V600E}\), as another cell line with BRAF\(^{V600E}\), 8505C, has been shown to respond to sorafenib and irradiation [12, 31].

The present study also investigated the role of autophagy in thyroid cancer treatment by comparing the effects of anticancer therapeutics between autophagy-competent and autophagy-defective cells. Additionally, mechanistic analysis of cell death induced by anticancer therapeutic modalities and autophagy inhibitors was conducted. The present data showing that the efficacy of anticancer therapeutics was significantly higher in autophagy-defective cells than autophagy-competent cells indicated the pro-survival effect of autophagy induced by anticancer therapeutics. The present study also demonstrated that anticancer therapeutics induced certain levels of apoptosis, which were further increased by autophagy inhibition. Thus, the mechanism for the pro-survival effect of autophagy induced by anticancer therapeutics is, at least in part, due to inhibition of apoptotic cell death. Indeed autophagy is generally considered to inhibit apoptosis [40]. Furthermore, addiction of cancer cells with BRAF\(^{V600E}\) to autophagy has been reported [41, 42], which is the most prevalent driver mutation in thyroid cancer.

It remains unclear why autophagy induced by irradiation was shown to be pro-survival in the present study and a previous study [25] but was anti-survival in a study by Lin et al. [31], even though all the groups used the same TPC1 cells. Regarding mTOR inhibitors, CZ415 induced autophagy and moderate levels of apoptotic cell death, the latter of which was augmented by autophagy induction with 3-MA (or short hairpinRNA-Beclin-1), indicating the pro-survival effect of autophagy induced by CZ415 [23]. In contrast, the experimental design in the study on RAD001 was considered peculiar, because the authors evaluated the effect of RAD001 on the cytotoxic effect of irradiation, that is, the effect of autophagy-inducing RAD001 on cytotoxicity of autophagy-inducing irradiation [26].

Some studies have reported the autophagy-independent anticancer mechanisms of autophagy inhibitors, CQ [43-45] and 3-MA [46]. Thus, caution should be taken when interpreting the data on the combined effect of anticancer therapeutics and autophagy inhibitors on cell death. However, a lack of the effect of combinations of sorafenib/irradiation with 3-MA/CQ in KTC1 cells, in which sorafenib and radiation show no effect on autophagy, strongly indicates that the combined effects are due to autophagy inhibition.

The limitations of the present study include the use of only three cancer cell lines, each of which has different mutational profiles. A future study with additional cell lines would be necessary to clarify the relationship between mutations and the response to autophagy inhibition. Furthermore, a definitive conclusion could be obtained from a clinical study that compares the therapeutic effectiveness in patients treated with anticancer therapeutics alone or in combination with autophagy inhibitors.

In conclusion, taking these limitations into consideration, the present study demonstrated, with four different types of anticancer therapeutics and three thyroid cancer cell lines, that autophagy induced by all of the anticancer therapeutics was shown to be pro-survival. Although the possibility for induction of autophagy-regulated cell death [7] by autophagy-inducing anticancer therapeutics cannot be completely excluded, and the fact that some cancer cells such as KTC1 cells may have a unique phenotype, the present study provides evidence for the beneficial combination of anticancer therapeutics with autophagy inhibitors, and proposes that autophagy inhibition may serve as a possible adjunctive therapy for thyroid cancer.

### Acknowledgement

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### Conflict of Interest

None of the authors have any potential conflicts of interest associated with this research.
Fig. S1  Effects of rapamycin, 3-MA, CQ, Sor, Cis, Dox and IR on cell viability, p62 expression and LC3 puncta in ACT1 cells. (A) Cell viability assay results. The cells were incubated with graded doses of chemicals or exposed to irradiation. After 48 h, cell viability was determined using the Cell Counting Kit-8 assay. (B and C) Immunofluorescence analyses of LC3 puncta and p62 expression. The cells were incubated for 48 h with 1 μM Rap, 5 mM 3-MA, 50 μM CQ, 10 μM Cis, 2 μg/mL Dox or 7 μM Sor, or exposed to IR (10 Gy). Quantitative analyses of LC3 puncta and p62 expression levels were performed. Original image magnifications: ×1,000 for LC3 and ×400 for p62. Data are presented as means ± SD. *, p < 0.05 vs. Cont. Rap, rapamycin; 3-MA, 3-methyladenine; CQ, chloroquine; Sor, sorafenib; Cis, cisplatin; Dox, doxorubicin; IR, irradiation; Cont, control.
**Fig. S2** Effects of Rap, 3-MA, CQ, Sor, Cis, Dox and IR on cell viability, LC3 punctate number and p62 expression in KTC1 cells. (A) Cell viability assay results. The cells were incubated with graded doses of chemicals or exposed to irradiation. After 48 h, cell viability was determined using the Cell Counting Kit-8 assay. (B) Immunofluorescence analyses of LC3 puncta and p62 expression. The cells were incubated for 48 h with 10 nM Rap, 1 mM 3-MA, 10 μM CQ, 1 μM Cis, 0.2 μg/mL Dox or 1 μM Sor, or exposed to IR (10 Gy). Quantitative analyses of LC3 puncta and p62 expression levels were conducted. (C) Dose-response experiments on the effect of Sor and IR on LC3 puncta and p62 expression. 100 nM to 2 μM Sor and 2 to 10 Gy IR were used. Original image magnifications, ×1,000 for LC3 and ×400 for p62. Data are presented as means ± SD. *, p < 0.05 vs. Cont. Rap, rapamycin; 3-MA, 3-methyladenine; CQ, chloroquine; Sor, sorafenib; Cis, cisplatin; Dox, doxorubicin; IR, irradiation; Cont, control; NS, not significant.
Fig. S3 Effects of the combinations of the different anticancer therapeutic agents with CQ on LC3 punctate number in ACT1 cells. (A) Immunofluorescence for LC3. The cells were treated with 7 μM Sor, 10 μM Cis or 2 μg/mL Dox, or exposed to 10 Gy IR alone or in combination with 50 μM CQ for 48 h. (B) Numerical analysis of LC3 puncta. The number of LC3 puncta was quantified using ImageJ software. Original image magnification, ×1,000. Data are presented as means ± SD. *; p < 0.05 vs. to Cont. CQ, chloroquine; Sor, sorafenib; Cis, cisplatin; Dox, doxorubicin; IR, irradiation; Cont, control; NS, not significant.

Fig. S4 Effect of the combination of Dox with CQ on LC3 punctate number in KTC1 cells. (A) Immunofluorescence for LC3. The cells were treated with 0.2 μg/mL Dox alone or in combination with 10 μM CQ for 48 h. Original image magnifications, ×1,000. (B) Numerical analysis of LC3 puncta. The number of LC3 puncta was quantified using ImageJ software. Data are presented as means ± SD. CQ, chloroquine; Dox, doxorubicin; IR, irradiation; NS, not significant.
Fig. S5 Kinetics of the last-step of autophagy induced by amino acid depletion and anticancer therapeutic agents in ACT1 cells. (A) Live-cell imaging of autolysosomes stained with DALGreen. The cells were stained with 1 μM DALGreen and then were either exposed to 10 Gy IR or incubated with one of the following anticancer drugs: 7 μM Sor, 10 μM Cis or 2 μg/mL Dox for up to 48 h. AA-free was used as a positive control. Original image magnifications, ×200. (B) The kinetics of autolysosome formation were quantified and expressed as arbitrary units. Data are presented as means ± SD. *; \( p < 0.05 \) vs. Cont. AA-free, amino acid-free medium; CQ, chloroquine; Sor, sorafenib; Cis, cisplatin; Dox, doxorubicin; IR, irradiation.

Fig. S6 Kinetics of the last-step of autophagy induced by amino acid depletion and different anticancer therapeutic agents in KTC1 cells. (A) Live-cell imaging of autolysosomes stained with DALGreen. The cells were stained with 1 μM DALGreen and then were either exposed to 10 Gy IR or incubated with 1 μM Sor for up to 48 h. AA-free was used as a positive control. Original image magnification, ×200. (B) The kinetics of autolysosome formation were quantified and expressed as arbitrary units. Data are presented as means ± SD. *; \( p < 0.05 \) vs. Cont. AA-free, amino acid-free medium; CQ, chloroquine; Sor, sorafenib.
Fig. S7 Effect of anticancer therapeutic agents alone or in combination with autophagy inhibitors 3-MA and CQ on viability of ACT1 cells. (A) Cell viability assay results. The cells were incubated with 1 μM Rap, 7 μM Sor or 10 μM Cis, or exposed to 10 Gy IR alone or in combination with 5 mM 3-MA or 50 μM CQ. Cell viability was determined using Cell Counting Kit-8 assay 48 h later. (B) Clonogenic assay results. The cells were treated with 5 mM 3-MA or 50 μM CQ for 24 h or left untreated, and then exposed to 2 Gy IR. After 7 days, the cells were stained and the number of colonies was counted. Representative images are shown in the left panel, and number of colonies was quantified manually (n = 3) and is shown in the right panel. All data are presented as means ± SD. *, p < 0.05. CQ, chloroquine; Sor, sorafenib; IR, irradiation; Rap, rapamycin; Cis, cisplatin; 3-MA, 3-methyladenine.

Fig. S8 Effect of anticancer therapeutic agents alone or in combination with autophagy inhibitors 3-MA and CQ on viability of KTC1 cells. The cells were incubated with 10 nM Rap, 1 μM Sor or 10 μM Cis, or exposed to 10 Gy IR alone or in combination with 5 mM 3-MA or 50 μM CQ. Cell viability was determined using the Cell Counting Kit-8 assay 48 h later. The data are presented as means ± SD. *, p < 0.05. CQ, chloroquine; Sor, sorafenib; IR, irradiation; Rap, rapamycin; Cis, cisplatin; 3-MA, 3-methyladenine; NS, not significant.
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