Research Paper

RITA plus 3-MA overcomes chemoresistance of head and neck cancer cells via dual inhibition of autophagy and antioxidant systems

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ABSTRACT

Reactivation of p53 and induction of tumor cell apoptosis (RITA) is a small molecule that blocks p53–MDM2 interaction, thereby reactivating p53 in tumors. RITA can induce exclusive apoptosis in cancer cells independently of the p53 pathway; however, the resistance of cancer cells remains a major drawback. Here, we found a novel resistance mechanism of RITA treatment and an effective combined treatment to overcome RITA resistance in head and neck cancer (HNC) cells. The effects of RITA and 3-methyladenine (3-MA) were tested in different HNC cell lines, including cisplatin-resistant and acquired RITA-resistant HNC cells. The effects of each drug alone and in combination were assessed by measuring cell viability, apoptosis, cell cycle, glutathione, reactive oxygen species, protein expression, genetic inhibition of p62 and Nrf2, and a mouse xenograft model of cisplatin-resistant HNC. RITA induced apoptosis of HNC cells at different levels without significantly inhibiting normal cell viability. Following RITA treatment, RITA-resistant HNC cells exhibited a sustained expression of other autophagy-related proteins, overexpressed p62, and displayed activation of the Keap1-Nrf2 antioxidant pathway. The autophagy inhibitor 3-MA sensitized resistant HNC cells to RITA treatment via the dual inhibition of molecules related to the autophagy and antioxidant systems. Silencing of the p62 gene augmented the combined effects. The effective antitumor activity of RITA plus 3-MA was also confirmed in vivo in mouse xenograft models transplanted with resistant HNC cells, showing increased oxidative stress and DNA damage. The results indicate that RITA plus 3-MA can help overcome RITA resistance in HNC cells.

Condensed abstract: This study revealed a novel RITA resistant mechanism associated with the sustained induction of autophagy, p62 overexpression, and Keap1-Nrf2 antioxidant system activation. The combined treatment of RITA with the autophagy inhibitor 3-methyladenine overcomes RITA resistance via dual inhibition of autophagy and antioxidant systems in vitro and in vivo.

1. Introduction

Head and neck cancer (HNC) is the eighth most common cancer worldwide, with more than a half a million new cases diagnosed each year [1]. The most common HNC pathology is squamous cell carcinoma, which commonly arises in the upper aerodigestive tract of the oral/nasal cavity, pharynx, and larynx [1,2]. A multidisciplinary approach consisting of surgery, radiotherapy, and chemoradiotherapy is frequently used for HNC treatment. Currently, radiotherapy and systemic chemotherapy are increasingly used for HNC treatment as an organ-preserving treatment strategy [3,4]. Cisplatin is used as a first-line agent in primary or postoperative chemoradiotherapy, often in combination with other anticancer chemotherapeutic agents [5]; however, chemotherapy is highly associated with acquired resistance and increased toxicity [6]. Epidermal growth factor receptor (EGFR) inhibitors and other molecular targeted agents have been used to overcome HNC resistance, but achieved only modest success in recurrent or metastatic HNC [7]. Despite recent therapeutic advances, the survival of patients with resistant HNC remains poor [4,8]. Moreover, prognostic improvement might be achieved by identifying methods of overcoming mechanisms of resistance and the identification of novel predictive biomarkers [7]. Furthermore, a new approach to circumventing chemotherapy resistance and to finding more effective anticancer agents is urgently needed for improving the treatment outcome.
for patients with HNC [9].

Recent studies have demonstrated a potential association between therapeutic resistance and autophagy. Chemotherapy agents, including cisplatin, may enhance autophagy in various types of human cancers; thus, a treatment approach that inhibits autophagy may reverse the observed chemoresistance [10–12]. In HNC patients, cetuximab has demonstrated only the limited success, with response rate under 20% [13]. The susceptibility of cancer cells to EGFR-targeted therapy may be modulated by autophagic responses, with a poor response to cetuximab therapy [14]. Furthermore, an autophagy blockade sensitizes HNC cells to EGFR inhibition and this approach combined with autophagy inhibition may enhance therapeutic efficacy [14]. Several molecular mechanisms related to autophagy and the therapeutic resistance of HNC cells have been reported [14–16].

Various stresses can trigger autophagy, involving the activation of p53, FOXO, MIT/TFE, Nrf2, and NF-κB/Rel families; moreover, signaling from autophagy modulates the stress response, including oxidative stress responses, via implementing negative feedback or positive feed-forward loops [17]. Of the autophagic machinery, p62/sequestosome 1 (SQSTM1) hereafter referred to as p62 involves the interaction between cytoprotective antioxidant pathways and autophagy via the translocation of autophagic cargo [18] and the activation of the Keap1-Nrf2 antioxidant response elements (ARE) pathway during selective autophagy [19]. In addition, p62 expression increases with enhanced autophagic flux, and high levels of p62 is associated with a poor therapeutic response and prognosis [20]. Therefore, a better understanding of the role of autophagy in anti-cancer therapy may contribute to overcome therapeutic resistance and predicting outcomes of cancer patients [21].

The reactivation of p53 and induction of tumor cell apoptosis (RITA) is a small molecule that directly interacts with p53 and induces a conformational change that prevents the interaction between p53 and MDM2 [22]. RITA treatment can lead to the apoptotic cell death of resistant cancer cells through restoring the function of p53 [23]. The anti-tumor activity of RITA has been also suggested to be an effective treatment strategy for multiple human cancers, even resistant cancer types with abnormal TP53, and functions independently of the p53 pathway [24–26]. Another potential application of RITA may be enhancing cisplatin cytotoxicity [27] and senescence [28] in HNC cells; however, RITA-induced autophagy protects cancer cells from apoptosis by inducing the phosphorylation of AMPK at Thr172 [29]. Furthermore, the anti-tumor activity of RITA decreases with the phosphorylation of NF-κB/RelA/p65 at Ser536 [30]. Thus, further studies are required to identify the mechanisms of RITA resistance in cancer cells, and facilitate the implementation of novel approaches to overcome this resistance. In the present study, we identified a novel mechanism of resistance to RITA treatment and an effective combinatorial agent that could overcome RITA resistance in HNC cells. In particular, protective autophagy and p62 overexpression contribute to RITA resistance, in conjunction with the activation of the Keap1-Nrf2-ARE antioxidant pathway. Furthermore, the combination of the autophagy inhibitor 3-methyladenine (3-MA) with RITA can overcome this resistance via the dual inhibition of autophagy and antioxidant system.

2. Materials and methods

2.1. Cell lines

This study used several HNC cell lines of AMC-HN2–10 previously established in our institute and SNU cell lines (SNU-1041, -1066, and -1076) purchased from the Korea Cell Line Bank (Seoul, Republic of Korea). All cell lines used in our studies were authenticated by short tandem repeat-based DNA fingerprinting and multiplex polymerase chain reaction (PCR). The cells were cultured in Eagle’s minimum essential medium or Roswell Park Memorial Institute 1640 (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO2. Normal oral keratinocytes (HOK) or fibroblasts (HOF) were obtained from patients undergoing oral surgery and were used for in vitro cell viability assays. The cisplatin-resistant and RITA-resistant HNC cell lines (HN4-cisR and HN4-ritaR) were developed from cisplatin-sensitive and RITA-sensitive parental HN4 cells, via continuous exposure to increasing cisplatin and RITA concentrations, respectively. The half maximal inhibitory concentrations (IC50) of cisplatin, determined by using cell viability assays, were 2.6 μM in HN4 and 25.5 μM in HN4-cisR cells, and the IC50 of RITA were 0.35 μM in HN4 and 20.6 μM in HN-ritaR cells.

2.2. Cell viability, cell cycle, and cell death assays

Cell viability after exposure to RITA (Cayman Chemical, Ann Arbor, MI, USA), 3-MA (Sigma-Aldrich, St. Louis, MO, USA), or its combinations for 72 h was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazolotriazolium, Sigma-Aldrich), trypan blue exclusion, and clonogenic assays. Control cells were exposed to an equivalent amount of dimethyl sulfoxide (DMSO). MTT assays were performed with the tetrazolium compound for 4 h, followed by a solubilization buffer for 2 h, and absorbance was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Trypan blue exclusion was performed with 0.4% trypan blue staining and counting using a hemocytometer. Clonogenic assays were performed with a 0.5% crystal violet solution and enumerating the number of colonies (> 50 cells) cultured for 14 days.

The cell cycle assay was performed after the cells had been treated with the indicated drugs for 72 h and then trypsinized, fixed in ice-cold ethanol, and stained for 30 min with propidium iodide (Sigma-Aldrich) at 37 °C. The cellular DNA content was measured using a FACScanCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). A cell death assay was also performed using staining with Annexin V and propidium iodide (PI) (Sigma-Aldrich) and then counting the number of Annexin V or PI-positive cells with flow cytometry and Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA). To measure the mitochondrial membrane potential (∆Vm), the cells were stained with 200 nM tetramethylrhodamine ethyl ester (TMRE, Thermofisher Scientific) for 20 min and analyzed by flow cytometry. The median fluorescent intensity (MFI) of each treatment group was normalized to the control group. All assays were performed with triplicate samples and repeated three times.

The interaction of two drugs was considered synergistic when growth suppression was greater than the sum of the suppression induced by either drug alone. CI of the drug interaction was scored using a software program (CombioSyn, Inc., Paramus, NJ, USA) and calculated using the Chou-Talalay method that defines CI < 1, synergistic interaction; CI = 1, additive interaction; and CI > 1, antagonistic interaction [31].

2.3. Glutathione synthesis and ROS production measurement

Cellular glutathione (GSH) levels were measured in the lysates of HNC cells exposed to different drugs for 24 h using a GSH colorimetric detection kit (BioVision Inc., Milpitas, CA, USA). Additionally, 2,7′-dichlorofluorescein diacetate (DCF-DA) (cytosolic ROS; Enzo Life Sciences, Farmingdale, NY, USA) was used to measure the level of cellular ROS generation in the supernatants of the HNC cell lysates treated for 24 h. The ROS levels were analyzed using a FACS Calibur flow cytometer equipped with CellQuest Pro software (BD Biosciences).

2.4. RNA interference

For silencing of the SQSTM1(p62) and NFE2L2 (Nrf2) genes, cisplatin-resistant HN4-cisR cells were seeded and transfected 24 h later with 10 nmol/L small interfering RNA (siRNA) targeting human SQSTM1, NFE2L2, or scrambled control siRNA (TriFECTa® RNAi kits;
2.5. Immunoblotting and Western blotting using anti-p62 and anti-Nrf2 antibodies.

Cells were lysed at 4 °C in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific). A total of 50 μg protein was resolved by SDS-PAGE on 10–12% gels, transferred to nitrocellulose or polyvinylidene difluoride membranes, and probed with primary and secondary antibodies. The following primary antibodies were used: poly(ADP-ribose) polymerase (PARP) and cleaved PARP, cleaved caspase 3, cleaved caspase 9, beclin-1, Atg5, p62, LC3, Bax, Bcl-2, Nrf2, heme oxygenase-1 (HO-1), (ADP-ribose) polymerase (PARP) and cleaved PARP, cleaved caspase 3, beclin-1, atg5, and LC3 proteins in RITA-sensitive (HN4 and HN9) and -resistant (HN6 and HN7) cells at 24 h after exposure to 5 μM RITA. (D) Immunofluorescence staining of LC3 (red color) in RITA-sensitive HN4 cells before (NT) and after treatment with 5 μM RITA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Immunoblotting

Cells were plated, grown to 70% confluence, and then subjected to treatment with the indicated drugs. The cells were also treated with Wortmannin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or chloroquine (Sigma-Aldrich) for 24 h, combined with or without RITA. Cells were lysed at 4 °C in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific). A total of 50 μg protein was resolved by SDS-PAGE on 10–12% gels, transferred to nitrocellulose or polyvinylidene difluoride membranes, and probed with primary and secondary antibodies. The following primary antibodies were used: poly(ADP-ribose) polymerase (PARP) and cleaved PARP, cleaved caspase 3, beclin-1, Atg5, p62, LC3, Bax, Bcl-2, Nrf2, heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1) (Cell Signaling Technology, Danvers, MA); and RAD51, H2AX, glutamate-cysteine ligase (GCLC) catalytic subunit (GCLC) and modifier subunit (GCLM), (Abcam, Cambridge, UK). β-actin (Sigma-Aldrich) was used as a loading control. All antibodies were diluted to between 1:250 and 1:5000.

2.6. Immunofluorescence staining

The cells treated with the indicated drugs and the untreated controls were stained with LC3 and/or p62 antibodies. The 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) was used as a nuclear counterstain. The cells were fixed with 3.7% paraformaldehyde in prewarmed complete medium for 15 min at 37 °C. The fixed cells were then deparaffinized, rehydrated, and stained with the target and secondary antibodies. The stained cells were observed on a fluorescent microscope for imaging. The number of LC3-stained puncta per section was counted for 10 cells per group in a total of 100 cells [14]. Mitochondrial superoxide generation was measured by mitoSOX (Thermo Fisher Scientific) in live cells treated with the indicated drugs. The stained cells were also observed on a fluorescent microscope. The mean fluorescent intensity of each group was normalized to that of the control group.

2.7. Tumor xenograft

All animal study procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of our institution. Six-week-old athymic BALB/c male nude mice (nu/nu) were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). HN4-cisR cells were subcutaneously injected into the flank of nude mice. From the day that gross nodules were detected from tumor implants, the mice were subjected to an intraperitoneal injection of four different treatments: vehicle, RITA (10 mg/kg/daily) [32], 3-MA (25 mg/kg/daily) [33], or RITA plus 3-MA. Each group included 10 mice. The tumor size and weight of each mouse were measured twice a week, and the volume was calculated as (length × width2)/2. The mice were sacrificed on day 35, and the tumors were included 10 mice. The tumor size and weight of each mouse were measured twice a week, and the volume was calculated as (length × width2)/2. The mice were sacrificed on day 35, and the tumors were isolated and analyzed by cellular GSH measurement and immunofluorescence staining of γH2AX formation. Arbitrary fluorescence units (AFU) were compared among the differently treated tumors. The number of apoptotic bodies in the tumors was counted in a blind manner in 10 randomly selected high-power fields after performing an in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Promega, Fitchburg, WI, USA).

2.8. Statistical analysis

Data were presented as the mean ± standard error of the mean. The statistical significance of the differences between treatment groups was assessed by a Mann-Whitney U-test or an analysis of variance with a Bonferroni post-hoc test using SPSS version 23.0 statistical software (IBM, Armonk, NY, USA). Statistical significance was defined as a two-sided P value < 0.05.

3. Results

3.1. RITA induces apoptosis of HNC cells at different levels

RITA decreased the viability of HNC cells at different levels in a dose-dependent manner (Fig. 1A and B). The most sensitive cancer cell lines to RITA treatment, were found to be AMC-HN4 and -HN9 cells, whereas AMC-HN2, -HN6, and -HN7 were relatively less sensitive cell lines. RITA did not significantly decrease the viability of normal oral keratinocytes and fibroblasts up to 10 μM of RITA treatment. Of the HNC cell lines, HN4/HN9 and HN6/HN7 were selected as RITA-
resistant and RITA-sensitive cell lines, respectively, for comparison of their molecular expression. RITA increased the expression of cleaved PARP and decreased the expression of autophagy-related proteins (beclin-1, Atg5, and LC3) in RITA-sensitive HNC cells (HN4 and HN9). In contrast, RITA failed to induce increased cleavage of PARP and sustained or increased the expression of autophagy-related proteins in RITA-resistant HNC cells (HN6 and HN7) (Fig. 1C). The changes in the autophagy-related proteins before and after RITA treatment were confirmed by LC3 immunofluorescent staining and puncta counting in the RITA-sensitive and -resistant HNC cells (P < 0.01; Fig. 1D and Supplementary Fig S1A).

3.2. HNC cell sensitivity to RITA depends on the different levels of autophagy induction

Cisplatin-resistant HN4-cisR and RITA-resistant HN4-ritaR cells exhibited no significant changes in the level of cell viability or colony forming ability up to a concentration of 10 μM RITA treatment compared to the cisplatin- and RITA-sensitive HN4 parental cell line (Fig. 2A and B). RITA treatment induced apoptosis of the sensitive HN4 cells, whereas no significant death occurred in the resistant HN4-cisR or HN4-ritaR cells (Supplementary Fig S2). This finding was also confirmed via a cell cycle analysis by the change in the subG1 fraction of HN4 and HN4-cisR cells that received RITA treatment. As previously shown, the expression of cleaved PARP and autophagy-related protein increased and decreased, respectively, in the sensitive HN4 cells, which was not observed in the resistant HN4-cisR and HN4-ritaR cells (Fig. 2C). This was also confirmed via LC3 puncta counting of these cell lines (Fig. 2D and Supplementary Fig S1B). The sustained expression of autophagy-related proteins in the RITA-resistant HN4-cisR cells were also identified via Western blot in time-dependent manner, when compared with that in the RITA-sensitive HN4 cells (P < 0.01; Supplementary Fig S3).

3.3. RITA plus 3-MA inhibits growth and induces apoptosis of resistant HNC cells

The addition of 3-MA to RITA treatment significantly decreased the cell viability and growth of the resistant HN4-cisR and HN4-ritaR cells (combination index (CI) < 1.0, P < 0.01; Fig. 3A and C). The colony-forming ability was also significantly decreased in the resistant HNC cells following the RITA and 3-MA combination therapy (P < 0.01; Fig. 3C). Apoptosis assays revealed enhanced Annexin-V positive staining in the resistant HN4-cisR cells following combination treatment with RITA and 3-MA when compared with RITA or 3-MA treatment alone, as well as the control (P < 0.01; Fig. 3D).

3.4. RITA plus 3-MA induces autophagy, antioxidant enzyme inhibition, mitochondrial damage, and apoptosis

RITA and 3-MA combination treatment inhibited the expression of autophagy-related proteins (beclin-1, Atg5, LC-3, and p62) in the resistant HN4-cisR cells (Fig. 4A). However, LC3 protein expression was not significantly altered by 3-MA or RITA plus 3-MA (Fig. 4A–B). In addition, the combination treatment induced the increased expression of pro-apoptosis and DNA-damage response proteins (cleaved caspase 3 and γH2AX) (Fig. 4C). The cell cycle was also altered in response to the combination treatment with RITA and 3-MA, in conjunction with an increased subG1 fraction, which was reversed via pretreatment with N-acetyl-l-cysteine (NAC) (Supplementary Fig S4). Changes in protein expression were also confirmed in the primary RITA-resistant HN6 cells (Supplementary Fig S5). The mitochondrial membrane potential (ΔΨm) also changed in the resistant HNC cells treated with the RITA and 3-MA combination (Fig. 4D). In addition to the inhibition of p62, the Nrf2 and ARE proteins (GCLC, GCLM, HO-1, and NQO1) decreased following treatment with the RITA and 3-MA combination in a dose-dependent manner, while Keap1 was observed to increase (Fig. 5A). The levels of cellular ROS increased and GSH levels decreased with the combination of RITA and 3-MA, which was abrogated with the pretreatment of 3 mM acetyl-L-cysteine (NAC) (Supplementary Fig S4). Changes in protein expression were also confirmed in the primary RITA-resistant HN6 cells (Supplementary Fig S5). The mitochondrial membrane potential (ΔΨm) also changed in the resistant HNC cells treated with the RITA and 3-MA combination (Fig. 4D). In addition to the inhibition of p62, the Nrf2 and ARE proteins (GCLC, GCLM, HO-1, and NQO1) decreased following treatment with the RITA and 3-MA combination in a dose-dependent manner, while Keap1 was observed to increase (Fig. 5A). The levels of cellular ROS increased and GSH levels decreased with the combination of RITA and 3-MA, which was abrogated with the pretreatment of 3 mM...
NAC \( (< 0.01; \text{Fig. 5B, and Supplementary Fig S6})\). Silencing of p62 and/or Nrf2 genes decreased cancer cell viability, which was augmented with RITA and 3-MA combination treatment \( (P < 0.05; \text{Fig. 5D})\). In contrast, the addition of Wortmannin or chloroquine to the RITA treatment induced no increase of pro-apoptotic protein, PARP, cleavage or caspase 3, but increased the level of Nrf2 expression. Moreover, such conditions also elicited different inhibitory levels of the autophagy-related proteins, beclin-1, Atg5, and LC3 \( (\text{Fig. 5E})\).

3.5. RITA plus 3-MA inhibits resistant HNC growth in vivo

In mouse xenograft models implanted with HN4-cisR cells, RITA plus 3-MA synergistically suppressed in vivo tumor growth, compared with no inhibitory effect following RITA or 3-MA treatment alone \( (\text{Fig. 6A and B})\). The RITA and 3-MA combined treatment also inhibited the growth of resistant HNC cells in vivo via the significant depletion of glutathione \( (\text{Supplementary Fig S7})\), as well as an increase in the number of TUNEL-positive apoptotic bodies and \( \gamma \text{H2AX} \) formation in the tumor cells \( (\text{Figs. 6C, 6D, and Supplementary Fig S8})\). Changes in daily oral food intake and body weight were not significantly different the control, RITA, 3-MA, or RITA plus 3-MA groups \( (\text{data not shown})\). Histological examination of the vital organs did not reveal any significant differences between these groups \( (\text{Supplementary Fig S9})\).

4. Discussion

The findings of present study revealed a novel RITA resistance mechanism via autophagy and the antioxidant system enhanced by the upregulation of p62. RITA induces the apoptosis of HNC cells at different levels without significantly inhibiting normal cell viability. Moreover, RITA also induces the degradation or sustained induction of autophagy-related proteins differently among various HNC cells, indicating that RITA sensitivity is associated with such variation in the level of autophagy induction. The Nrf2-ARE antioxidant pathway that accompanies the induction of autophagy also contributes to the RITA resistance exhibited by HNC cells. In addition, our findings suggest a novel approach to overcome the chemoresistance related to RITA treatment. The combination of RITA with the autophagy inhibitor, 3-MA, can overcome the RITA resistance via the dual inhibition of autophagy and antioxidant system. This combined therapy increases the level of oxidative stress and DNA damage in resistant HNC cells, resulting in effective growth inhibition and elimination of cancer cells both in vitro and in vivo. Our study is the first to show that treatment with RITA plus 3-MA may be an effective method to overcome the RITA resistance of HNC cells.

Restoration of wild-type p53 function prompts the rapid elimination of human cancers carrying a functional loss of p53, which is frequently mutated in a large fraction of tumors \[34\]. In addition, p53-activating small molecules, such as PRIMA-1, RITA, and nutlin, have been shown to induce anti-tumor effects in various human tumors \[35\]. Moreover, a p53-targeting compound, PRIMA-1MET (APR-246), has achieved promising results in phase I/II clinical trials \[36\]. The small molecule, RITA, was developed as a new anti-cancer drug capable of binding p53, blocking the p53–MDM2 interaction, and leading to p53 activation in
tumor cells [22,23]. Furthermore, RITA can eliminate multiple types of human cancers independently of p53 function, including HNC and p53-defective cells, via the activation of other signaling pathways [24–26]. For example, RITA induces apoptosis through p38 and JNK/SAPK in tumor cells harboring mutant p53, p53-null, and wild-type p53 [37]. This suggests that RITA can be used as a promising small molecule that is able to kill cancer cells with mutant p53, relatively carrying the resistance to conventional chemotherapy [24–26].

Several recent studies have demonstrated that some human tumors are resistant to RITA treatment [29,30]. RITA regulates the mTOR pathway by inducing AMPK phosphorylation (Thr172); AMPK inhibitor (e.g., compound C) or autophagy inhibitors (e.g., chloroquine or 3-MA), sensitizes cancer cells to RITA-induced apoptosis [29]. The RITA chemosensitivity of cancer cells may also be dependent on site-differential phosphorylation of NF-κB RelA/p65 and can be restored via the silencing of ATP-binding cassette transporter ABCC6 overexpression [30]. Furthermore, a recent study found that the pharmacological inhibition of mTOR or the genetic inhibition of FancD2 restored RITA sensitivity in tumors exhibiting cross-resistance to DNA crosslinking compounds, such as cisplatin [38]. This effect was also supported by our finding that cisplatin-resistant HN4 cells include RITA resistance, even those harboring wild-type TP53. Moreover, the restoration of RITA sensitivity recovered the DNA damage response (increased γH2AX formation).

The current study also characterized a novel mechanism of RITA resistance in cancer cells. RITA-resistant HNC cells exhibited the sustained expression of autophagy-related proteins and the overexpression of p62. Increasing evidence has shown that of a variety of factors that contribute to drug resistance, autophagy is significantly associated with therapeutic resistance to multiple chemotherapeutic agents used against several types of human cancers [10–12] Our study closely investigated the importance of p62 overexpression as a resistance mechanism of RITA. Cytosolic overexpression of p62, in conjunction with other changes related to autophagy-related protein expression, is associated with aggressive features of tumors, an unfavorable response to therapy, and poor prognosis in glioblastoma [39], and breast [40], colon [41], and oral cancers [20]. In addition, p62 plays a critical role at the hub of signaling molecules involved in multiple cellular functions to control cell survival, apoptosis, and autophagy [42]. p62 is assembled in autophagic cargo, phosphorylated in an mTORC1-dependent manner, and subsequently induces the expression of the Keap1-Nrf2 antioxidant pathways [19]. Our study also demonstrated that p62 is involved in coupling the Keap1-Nrf2 system to autophagy, contributing to the RITA resistance observed in HNC cells. The increased expression of the Nrf2-ARE pathways induced by p62 and autophagy might further enhance the resistance of HNC cells toward chemotherapeutic agents, as the link between Nrf2 overexpression and therapeutic resistance has been previously reported [43,44]. Our study is the first to show that the dual activation of autophagy and antioxidant system may enhance the resistance to RITA treatment in HNC cells.

The induction of autophagy in response to chemotherapeutics may constitute a potential target for anticancer therapy, as well as drug resistance [45]. Resistance to receptor tyrosine kinase inhibitors is closely linked to autophagy in solid tumors, and blocking autophagy may be a promising strategy in fighting cancer [46]. The current study
showed that the combination of an autophagy inhibitor (3-MA) with RITA might be a promising approach to overcome the chemoresistance related to RITA treatment. RITA plus 3-MA treatment overcomes RITA resistance via the dual inhibition of autophagy and the Nrf2-ARE system commonly upregulated in chemo-resistant HNC cells. Inhibition of p62 expression and autophagy by RITA plus 3-MA decreases the activation of the Keap1-Nrf2 pathway. Furthermore, the inhibition of p62 promotes the accumulation of Keap1 protein and enhances Keap1-mediated Nrf2 degradation, suggesting that the p62-Keap1 interaction is responsible for Nrf2 expression against cancer cell death \[47\]. The combinatorial therapy sensitizes chemo-resistant HNC cells to RITA treatment through the dual inhibition of autophagy and antioxidant system, resulting in increased cellular oxidative stress and DNA damage response (Fig. 7). This dual inhibition leads to the depletion of the antioxidant, GSH, and markedly increases the level of cellular ROS in cancer cells.

The use of genetic inhibition or selective inhibitors of autophagy or the mTOR pathway have been suggested to overcome chemoresistance; however, there are conflicting results associated with the efficacy of 3-MA in terms of inhibiting autophagy and its related cancer resistance. Knockdown of ATG5 or treatment with rapamycin or hydroxychloroquine restores the sensitivity of tongue cancer cells to an EGFR kinase inhibitor, erlotinib, but 3-MA did not influence autophagy \[48\]. The use of 3-MA or siRNA targeting beclin 1 inhibited DHA-E3-induced autophagy and subsequently overcome P-glycoprotein-mediated multidrug resistance \[49\]. The degradation of LC3 or inhibition of LC3-I to LC3-II conversion by 3-MA treatment may be presented at various levels in cancer cells. Our study revealed no significant changes in LC3-I or LC3-II protein expression following 3-MA treatment in RITA-resistant cancer cells, in contrast to the significant decreases observed in the expression of other autophagy-related proteins. In addition, 3-MA effectively inhibited autophagy and decreased the expression of p62 and the Keap1-Nrf2-ARE antioxidant pathways in resistant HNC cells, compared to the changes induced by other autophagy inhibitors (e.g., Wortmannin and chloroquine). Therefore, the decreased cellular GSH levels and increased ROS stress induced by treatment with 3-MA plus RITA potentiated cancer cell death through oxidative stress and DNA damage; however, the efficacy and mechanisms of 3-MA combined with other chemotherapeutic agents require further study.
5. Conclusion

This study suggests that the induction of autophagy and overexpression of antioxidant system overexpression is involved in the mechanism of resistance to RITA therapy in HNC cells. The sustained expression of autophagy-related proteins and p62 is linked to the increased expression of Keap1-Nrf2, which consequently contributes to HNC resistance. Thus, the use of 3-MA may sensitize resistant HNC cells to RITA treatment via the dual inhibition of autophagy and antioxidant system, manifesting as enhanced oxidative stress and DNA damage in tumors both in vitro and in vivo. Further preclinical and clinical investigation of RITA and 3-MA should be performed in patients with resistant cancer types to explore this promising anti-cancer therapy.

Author contributions

Daiha Shin and Jong-Lyel Roh conceived and designed the experiments. Daiha Shin, Eun Hye Kim, Jaewang Lee, and Jong-Lyel Roh performed the experiments. Daiha Shin and Jong-Lyel Roh analyzed the data. Daiha Shin and Jong-Lyel Roh contributed reagents/materials/analysis tools; Daiha Shin and Jong-Lyel Roh wrote the draft, and checked and revised. All authors approved to submit this version to this publication.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.05.025.

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