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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 extremely urgent for improving the treatment outcome

Abbreviations: 3-MA, 3-methyladenine; ARE, antioxidant response elements; ATG5, autophagy related 5; CI, combination index; DCF-DA, 2′,7′-dichlorofluorescein diacetate; GSH, glutathione; HNC, head and neck cancer; IHC, immunohistochemistry; LC3, microtubule-associated protein 1A/1B-light chain 3; MiT/TFE, microphthalmia transcription factor; Nrf2, nuclear factor (erythroid-derived 2)-like 2; RITA, reactivation of p53 and induction of tumor cell apoptosis; ROS, reactive oxygen species; siRNA, short interfering RNA; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

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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% CO_2. 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 (IC_{50}) of cisplatin, determined by using cell viability assays, were 2.6 μM in HN4 and 25.5 μM in HN4-cisR cells, and the IC_{50}s 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-diphenyltetrazolium bromide, 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 FACSCalibur 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 (ΔΨm), the cells were stained with 200 nM tetramethylrhodamine ethyl ester (TMRE, Thermo Fisher 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 (ComboSyn, Inc., Paramus, NJ, USA) and calculated using the Chou-Talalay method that defined as 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 FACSCalibur flow cytometer equipped with CellQuest Pro (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;