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# Nrf2 inhibition reverses the resistance of cisplatin-resistant head and neck cancer cells to artesunate-induced ferroptosis

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ABSTRACT

Artesunate, an anti-malarial drug, has been repurposed as an anticancer drug due to its induction of cell death *via* reactive oxygen species (ROS) production. However, the molecular mechanisms regulating cancer cell death and the resistance of cells to artesunate remain unclear. We investigated the molecular mechanisms behind the antitumor effects of artesunate and an approach to overcome artesunate resistance in head and neck cancer (HNC). The effects of artesunate and trigonelline were tested in different HNC cell lines, including three cisplatin-resistant HNC cell lines. The effects of these drugs as well as the inhibition of Keap1, Nrf2, and HO-1 were assessed by cell viability, cell death, glutathione (GSH) and ROS production, protein expression, and mouse tumor xenograft models. Artesunate selectively killed HNC cells but not normal cells. The artesunate sensitivity was relatively low in cisplatin-resistant HNC cells. Artesunate induced ferroptosis in HNC cells by decreasing cellular GSH levels and increasing lipid ROS levels. This effect was blocked by co-incubation with ferrostatin-1 and a trolox pretreatment. Artesunate activated the Nrf2–antioxidant response element (ARE) pathway in HNC cells, which contributed to ferroptosis resistance. The silencing of Keap1, a negative regulator of Nrf2, decreased artesunate sensitivity in HNC cells. Nrf2 genetic silencing or trigonelline reversed the ferroptosis resistance of Keap1-silenced and cisplatin-resistant HNC cells to artesunate *in vitro* and *in vivo*. Nrf2–ARE pathway activation contributes to the artesunate resistance of HNC cells, and inhibition of this pathway abolishes ferroptosis-resistant HNC.

*Condensed abstract:* Our results show the effectiveness and molecular mechanism of artesunate treatment on head and neck cancer (HNC). Artesunate selectively killed HNC cells but not normal cells by inducing an iron-dependent, ROS-accumulated ferroptosis. However, this effect may be suboptimal in some cisplatin-resistant HNCs because of Nrf2–antioxidant response element (ARE) pathway activation. Inhibition of the Nrf2–ARE pathway increased artesunate sensitivity and reversed the ferroptosis resistance in resistant HNC cells.

## 1. Introduction

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide, accounting for an estimated 650,000 new cancer cases and 350,000 cancer deaths every year [1]. Squamous cell carcinoma consists of more than 90% of HNCs that arise in the essential organs responsible of respiration, swallowing, articulation and speech, including the oral/nasal cavity, larynx, and pharynx. HNC is commonly treated with the multimodal approach of surgery, radiotherapy, and systemic chemotherapy. Radiotherapy and chemotherapy are typically used to preserve the morphology and functionality of HNC-affected organs [2–4]. Cisplatin is used as a first-line chemotherapeutic agent in combination with radiotherapy in an organ preserving protocol for HNC [5]. However, cisplatin is commonly associated with acquired resistance and the toxicity of various organs in clinical settings, which leads to a failure in cancer patient management [6]. Therefore, a new approach to circumventing cisplatin resistance and toxicity is very urgent for the improved treatment of HNC [7]. Recently, the induction of regulated nonapoptotic cell death was presented as a useful strategy to eliminate cancer cells resistant to drug-induced apoptosis [8].

Artesunate is a water-soluble semi-synthetic derivative of artemisinin and a first-line treatment for malaria [9]. Artesunate has been repurposed as an anticancer drug that induces cell death by reactive

*Abbreviations:* HNC, head and neck cancer; GSH, glutathione; TrxR, thioredoxin reductase; Nrf2, nuclear factor erythroid-derived 2-like 2 (NFE2L2); Keap 1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; HO-1, heme oxygenase 1 (HMOX1); ROS, reactive oxygen species; DCF-DA, 2′,7′-dichlorofluorescein diacetate; MTT, 3-[4]-2,5-diphenyl-2H-tetrazolium bromide; siRNA, short interfering RNA; shRNA, short hairpin RNA

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oxygen species (ROS) production [10–13]. Artemisinins enhanced anticancer toxicity by utilizing ferrous iron to generate radicals that could kill cancer cells [14,15]. Artesunate also induces lysosomal ROS-mediated mitochondrial apoptosis and targets the DNA damage and repair systems in conventional chemotherapeutic drug-resistant cancer cells [16–20]. Furthermore, a recent report suggested that artesunate induced iron-dependent, ROS-producing mitochondrial stress and non-caspase apoptosis [21]. Recently, artesunate was identified as a specific activator of a novel iron-dependent, caspase-independent, nonapoptotic ferroptosis that killed resistant cancer cells with oncogenic KRAS reprogramming [22]. Artesunate has been tested for its anticancer effects on various types of human malignancies but rarely using HNC cells [12,14,16–19,21,23–25].

Ferroptosis is a new form of regulated cell death that is distinct from apoptosis, necroptosis, and autophagic cell death at morphological, biochemical, and genetic levels [26]. In addition, the inhibition of cystine/glutamate antiporter (xCT), a key molecule related to ferroptosis, may induce the eradication of cancer cell resistance to conventional radiotherapy or chemotherapy [27]. Recently, activation of the p62- Keap 1 (Kelch-like ECH-associated protein 1)-Nrf2 (nuclear factor erythroid 2-related factor 2) pathway was shown to determine the therapeutic response to ferroptosis-targeted therapies in cancer cells [28].

Our prior study suggested that cisplatin resistance in HNCs can be overcome by the induction of ferroptosis [29]. In addition, artesunate downregulates RAD51 and increases γH2AX formation, which results in sensitizing ovarian cancer cells to cisplatin [20]. However, the molecular mechanisms regulating cancer cell death and artesunate resistance remain unclear. The resistance of cancer cells to artesunate-induced ferroptosis has been largely unstudied in human cancers, including HNCs. Investigations on this topic will lead to a better molecular and therapeutic understanding of how artesunate overcomes HNC resistance to conventional chemotherapy. Here we investigated the molecular mechanisms of artesunate-based antitumor effects and resistance and identified an approach for overcoming HNC resistance. Activation of the Nrf2-antioxidant response element (ARE) pathway contributed to the ferroptosis resistance of cisplatin-resistant HNC cells and a combined therapy targeting the Nrf2-ARE pathway with artesunate eliminated HNC resistance *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Cell lines

HNC cell lines (HN2–10) and SNU cell lines were purchased from the Korea Cell Line Bank (Seoul, Republic of Korea) and 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 (RPMI) 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 or fibroblasts were obtained from patients undergoing oral surgery and used for *in vitro* assays. Three cisplatin-resistant HNC cell lines (HN3-cisR, HN4-cisR, and HN9-cisR) were developed from the parental HN3, HN4, and HN9 cells, respectively, by subjecting them to continuous exposure of increasing cisplatin concentrations [30]. The half-maximal inhibitory concentrations ($IC_{50}$) of cisplatin (Sigma-Aldrich, St. Louis, MO, USA) in the parental (2.2–3.5 μM) and cisplatin-resistant (25.5–38.9 μM) HNC cells were determined using cell viability assays.

### 2.2. Cell viability assays

Cell viability after exposure to artesunate (1–100 μM, Sigma-Aldrich) and/or trigonelline (100 μM, Sigma-Aldrich) with or without Nrf2 genetic inhibition and activation was assessed using MTT, trypan blue exclusion, and clonogenic assays. For the MTT assay, cells were incubated with the tetrazolium compound MTT (3-[4]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich) for 4 h, followed by a solubilization buffer for 2 h. Next, the absorbance was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The trypan blue exclusion assay was performed with 0.4% trypan blue staining, and cells were counted using a hemocytometer. The clonogenic assay was performed with a 0.5% crystal violet solution and quantification of the number of colonies ( > 50 cells) after 14 days in culture. A cell death assay was performed using annexin V and propidium iodide (PI) (Sigma-Aldrich) staining. Positively stained cells were counted using flow cytometry and Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA). All assays were performed with triplicate samples and repeated three times. The combination index (CI) of drug interaction was calculated using the Chou–Talalay method with a CI < 1 defined as a synergistic interaction (ComboSyn, Inc., Paramus, NJ, USA) [31].

### 2.3. Glutathione synthesis and ROS measurement

Cellular GSH levels were measured in the lysates of cells exposed to different drugs for 24 h using a GSH colorimetric detection kit (BioVision Inc., Milpitas, CA, USA). Cellular ROS generation in the supernatant of HNC cell lysates treated for 24 h was measured by adding 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) (cytosolic ROS; Enzo Life Sciences, Farmingdale, NY, USA) or 2 μM C11-BODIPY C11 (lipid peroxidation; Thermo Fisher Scientific) for 30 min at 37 °C. The ROS levels were analyzed using a FACSCalibur flow cytometer equipped with CellQuest Pro (BD Biosciences).

### 2.4. RNA interference and gene transfection

For the silencing of the *KEAP1* gene, cisplatin-sensitive HN9 cells were seeded. For the silencing of *NFE2L2* and *HMOX1* genes, cisplatin-resistant HN3- and HN9-cisR cells were seeded. Cells were transfected 24 h later with 10 nM small interfering RNA (siRNA) or scrambled control siRNA (Integrated DNA Technologies, Coralville, IA, USA). For stable *KEAP1* or *NFE2L2* knockdown, the HN9-cisR cells were transfected with a lentiviral vector containing small hairpin RNA (shRNA) directed against *KEAP1* or *NFE2L2* or control shRNA (Transomic, Huntsville, AL, USA). Cells with a stable transfection were selected using 2 μg/mL puromycin (Sigma-Aldrich). The siRNA or shRNA-induced gene silencing was confirmed using Western blotting and reverse transcription–quantitative PCR (RT–qPCR) from 1 to 2 μg of total RNA from each sample using the SuperScript® III RT-PCR system (Thermo Fisher Scientific).

### 2.5. Western blotting

Cells were plated, grown to 70% confluence, and then subjected to treatment with the indicated drugs. Cells were lysed at 4 °C in a radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific). A total of 50 μg of 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: Nrf2, Keap1, NQO1, HO-1 (Abcam, Cambridge, UK), xCT, p53, RAD51, and CD44 (Cell Signaling Technology, Danvers, MA). β-actin (Sigma-Aldrich) was used as a loading control. All of the antibodies were diluted between 1:500 and 1:5000.

### 2.6. Nrf2 transcriptional activity assay

The transcriptional activity of Nrf2 was assayed using a Cignal Antioxidant Response Reporter kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.