

## RESEARCH ARTICLE

# Sulfasalazine Induces Autophagic Cell Death in Oral Cancer Cells via Akt and ERK Pathways

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### Abstract

Sulfasalazine (SSZ) is an anti-inflammatory drug that has been used to treat inflammatory bowel disease and rheumatoid arthritis for decades. Recently, some reports have suggested that SSZ also has anti-cancer properties against human tumors. However, little is known about the effects of SSZ on oral cancer. The aim of this study was to investigate the anti-cancer effects of SSZ in oral squamous cell carcinoma (OSCC) cells and to elucidate the mechanisms involved. The authors investigated the anti-proliferative effect of SSZ using the MTT method in HSC-4 cells (an OSCC cell line). Cell cycle analysis, acidic vesicular organelle (AVO) staining, monodansylcadaverine (MDC) staining and Western blotting were also conducted to investigate the cytotoxic mechanism of SSZ. SSZ significantly inhibited the proliferation of HSC-4 cells in a dose-dependent manner. In addition, SSZ induced autophagic cell death, increased microtubule-associated protein 1 light chain (MAP1-LC; also known as LC) 3-II levels, as well as induced punctate AVO and MDC staining, resulted in autophagic cell death. Furthermore, these observations were accompanied by the inhibition of the Akt pathway and the activation of ERK pathway. These results suggest that SSZ promotes autophagic cell death via Akt and ERK pathways and has chemotherapeutic potential for the treatment of oral cancer.

**Keywords:** Sulfasalazine - oral cancer - autophagic cell death - Akt - ERK

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### Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity, and the sixth most frequently diagnosed cancer worldwide (Jemal et al., 2011; Siegel et al., 2012). Most OSCC patients are usually diagnosed after the cancer has reached an advanced stage, and thus, this tumor type is associated with poor prognosis. Indeed, the 5-year survival rate of OSCC patients remains extremely low despite of improvements in OSCC therapy. Furthermore, some chemotherapeutic agents such as taxol, 5-FU, and cisplatin, which have been used to treat OSCC, have associated with side effects, which include immunosuppression, nausea/vomiting, oral ulcers, and hair loss. Therefore, it is clear that the development of novel therapeutic agents against OSCC is necessary.

Sulfasalazine (SSZ) is composed of sulfapyridine and mesalazine (mesalamine, 5-aminosalicylic acid). It is an anti-inflammatory drug that has been used routinely for decades to treat inflammatory bowel disease and rheumatoid arthritis (Ogrendik, 2013). The therapeutic effects of SSZ are primarily attributed to mesalazine, which has favorable side-effect profile (Ogrendik, 2013).

Recent studies found that SSZ induces the apoptosis in several human cancer cells, including esophageal cancer, lung cancer and glioblastoma cells (Robe et al., 2004; Lay et al., 2007; Li et al., 2009). Recently, Guo et al. (2011) have reported that SSZ can inhibit cell growth in hepatocellular carcinoma via autophagy. However, the chemotherapeutic potential of SSZ in OSCC has yet to be elucidated.

Autophagy, also called type II programmed cell death, is an intrinsic self-digestive process that occurs in response to various pathologic conditions and environmental stressors such as developmental processes, infectious agents, cancer, neurodegeneration and aging (Liu et al., 2011; Banjerdpongchai and Khaw-On, 2013; Du et al., 2013). Although autophagy has been considered to be a cellular protective mechanism against cytotoxic agents (Eisenberg-Lerner and Kimchi, 2009), controversies regarding the roles of autophagy have recently emerged. Some researchers have suggested that the initiation of autophagy in response to chemotherapeutic agents functions as a cell death mechanism (Pandey and Chandravati, 2012). Moreover, crosstalk between autophagy and apoptosis can be complex and has

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seemingly contradictory roles that depend on the target cancer cell line or drug. In particular, autophagy can inhibit or promote apoptosis, or autophagy and apoptosis can co-occur in damaged cancer cells.

In this study, we investigated the type of cell death induced by SSZ in HSC-4 cells (OSCC cell line). In addition, we sought to identify the molecular mechanisms that underlie SSZ-induced autophagy in HSC-4 cells and evaluated the chemotherapeutic potential of SSZ.

## Materials and Methods

### Reagents and antibodies

Sulfasalazine, paclitaxel, MTT (3,4,5-dimethyl N-methylthiazol-2-yl-2,5-d-phenyl tetrazolium bromide), acridine orange (used for acidic vesicular organelle (AVO) staining), monodansylcadaverine (MDC), primary antibodies against microtubule-associated protein 1 light chain (MAP1-LC; also known as LC3), 3,3-Methyladenine (3-MA) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against phospho-ERK, phospho-Akt, and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against beta-actin, ERK, and secondary antibodies were purchased from Santa Cruz (CA, USA), and antibody against Atg 5-12 was obtained from Abcam (Cambridge, UK).

### Cell culture

HSC-4 cells, one of oral squamous cell carcinoma (OSCC) cell line, were maintained in medium composed of DMEM and Ham's Nutrient Mixture F-12 (Gibco, BRL, USA) at a ratio of 3:1, supplemented with insulin (Sigma-Aldrich), apo-transferrine (Sigma-Aldrich), triiodothyronine (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), cholera toxin (Sigma-Aldrich), 10% FBS (Hyclone, UT, USA) and 1% penicillin/streptomycin (Invitrogen, NY, USA) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

### MTT assay

HSC-4 cells were seeded in 24-well plates (5×10<sup>4</sup> per well) and incubated overnight. Cells were then treated with DMSO (vehicle) or 0.5, 1.0, or 1.5 mM of SSZ for 24 h. To evaluate the viability of SSZ-treated cells, a working solution of MTT was added to each well and cells were incubated at 37°C for an additional 4 h. Absorbances (570 nm) were measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

### Cell morphology

To examine morphological changes of HSC-4 cells after SSZ treatment, photographs were taken using a phase contrast microscope at the magnification, X200 (Olympus, Tokyo, Japan).

### Cell cycle analysis

Cell cycle analysis was performed to determine the nature of SSZ-induced cell death. Briefly, HSC-4 cells (1×10<sup>6</sup> cells per well) were treated with DMSO (vehicle) or 0.5, 1.0, or 1.5 mM of SSZ for 24 h. Cells were then

collected, centrifuged at 2000 rpm for 5 min, washed in PBS, and fixed with 75% EtOH. PI solution (10 µg/ml) was added at room temperature prior to cell cycle distribution analysis, which was conducted using a FACS Scan flow cytometer (BD Biosciences, Heidelberg, Germany).

### AVO staining

AVO staining was used to detect the presence of acidic vesicles after SSZ treatment. Cells were treated with a final concentration of 1 µM of acridine orange solution at 37°C for 15 min, washed in PBS and observed under a fluorescence microscope (excitation=488 nm, emission=520 nm) (Carl Zeiss, Germany).

### MDC staining

MDC staining was performed to detect autophagosomes and autophagic vacuoles. Briefly, HSC-4 cells were seeded on round coverslips in 24-well plates and treated with DMSO (vehicle) or 0.5, 1.0, or 1.5 mM of SSZ, and then with 0.05 mM of MDC for 15 min at 37°C. The images were captured using a fluorescence microscope at the magnification of 400 (excitation=360 nm, emission=525 nm) (Carl Zeiss, Germany).

### Western blot analysis

SSZ-treated HSC-4 cells were collected, washed, and lysed with RIPA buffer (Cell Signaling Technology) for 30 min. A Bradford assay was used to determine the protein concentration of the cell lysate supernatants. SDS-PAGE gels were loaded with 50 µg of protein per well and separated proteins were transferred to polyvinylidene fluoride membrane. The membranes were incubated with primary antibodies targeting LC3, Atg5-12, Akt, phospho-Akt, ERK, phospho-ERK and beta-actin overnight at 4°C, and incubated with the appropriate HRP-conjugated secondary antibody. Antigen-antibody complexes were visualized using SuperSignal West-Femto reagent (1:8000 dilution; Pierce, Rockford, IL, USA).

### Statistical analysis

All data are presented as means±standard errors. For statistical analysis, we used the student's t-test in Windows PASW (Predictive Analytics SoftWare) version 21.0 (SPSS Inc., NY, USA). P values of <0.05 were regarded statistically significant.

## Results

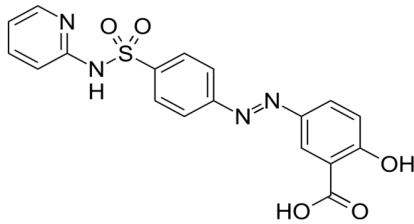
### SSZ inhibited the proliferation of HSC-4 cells and also altered cellular morphology

SSZ treatment with over 0.5 mM significantly inhibited the proliferation of HSC-4 cells in a dose-dependent manner (Figure 2A) with IC<sub>50</sub> of 1.25 mM. In addition, after treatment with DMSO (vehicle) or 0.5, 1.0, or 1.5 mM of SSZ for 24 h, cell size reduction, the increase of cell granularity and numerous intracellular vacuoles were observed (Figure 2B).

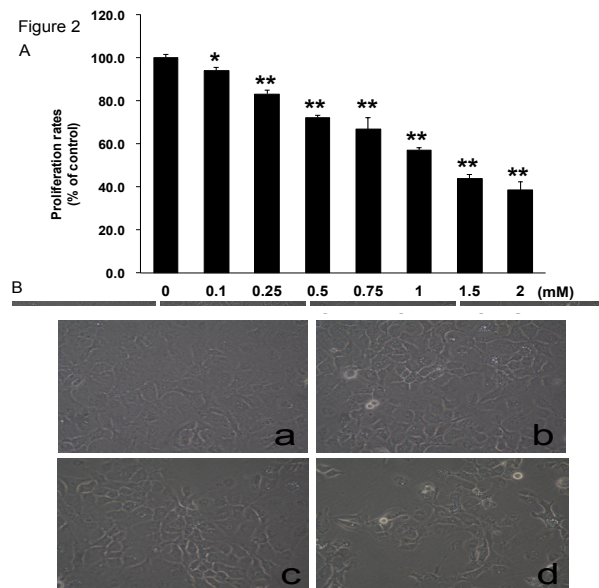
### The proportions of cells undergoing sub-G1 arrest were slightly increased in SSZ-treated HSC-4 cells

To identify the type of cell death induced by SSZ, we

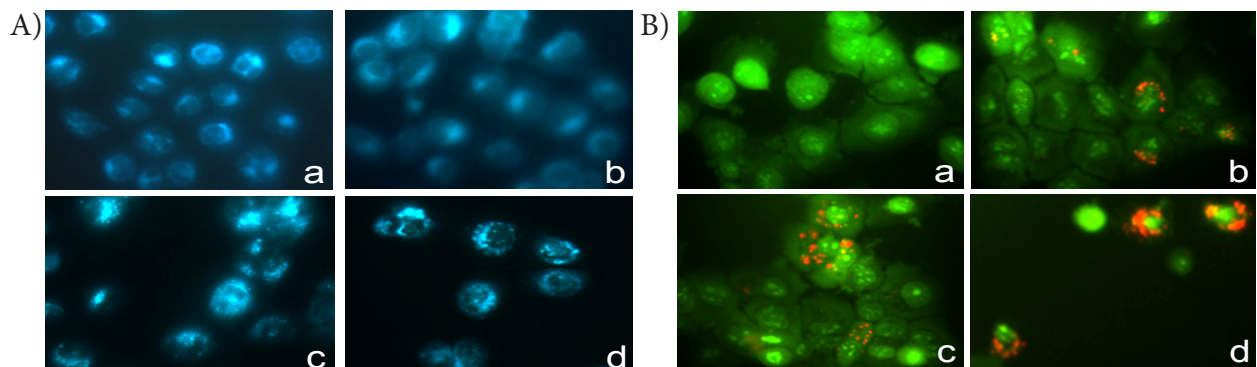
analyzed cell cycle. SSZ was found to induce sub-G1 arrest in HSC-4 cells, for example, treatment with SSZ at 1.5 mM induced sub-G1 arrest in 11.0% of cells as compared with 2.5% of cells in vehicle treated-cells (Figure 3).



**Figure 1. Chemical Structure of SSZ**



**Figure 2. Effects of SSZ on the Proliferation and Morphological Change in HSC-4 Human Oral Cancer Cells.** After treatment with various concentrations of SSZ for 24 h, proliferation rates were determined using a MTT assay. Results were presented as means±standard deviations \* $p < 0.01$ , \*\* $p < 0.001$  A) Proliferation rates of HSC-4 cells. B) Morphological changes exhibited by SSZ-treated HSC-4 cells at concentrations of (a) 0 mM; (b) 0.5 mM; (c) 1.0 mM; and (d) 1.5 mM. Cell morphologies were observed under a phase-contrast microscopy (X200)



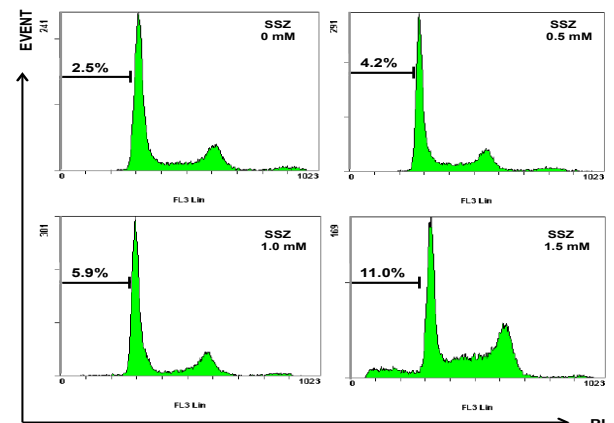
**Figure 4. Effects of SSZ on the Formations of Acidic Vesicular Organelles and Autophagic Vacuoles in HSC-4 Cells.** Cells were treated with vehicle or 0.5, 1.0, or 1.5 mM of SSZ for 24 h, then cells were stained with acridine orange or monodansylcadaverine (MDC) and visualized by fluorescent microscopy. A) AVO staining at SSZ concentrations of (a) 0 mM; (b) 0.5mM; (c) 1.0mM; and (d) 1.5mM. B) MDC staining at SSZ concentrations of (a) 0 mM; (b) 0.5mM; (c) 1.0mM; and (d) 1.5mM (Original magnification, X400)

#### Induction of autophagy in SSZ-treated HSC-4 cells as determined by AVO and MDC staining

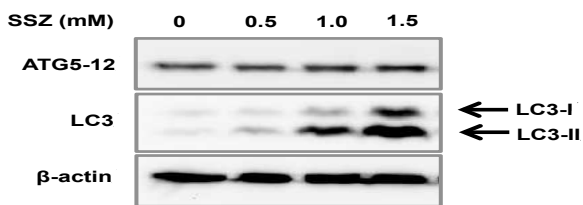
As shown in Figure 4A, in the vehicle-treated group, AVO formation was not been detected, but distinct dose-dependent punctate formation (Figure 4A), which is indicative of autophagy induction was observed in SSZ-treated HSC-4 cells. MDC staining was no different in vehicle-treated cells and 0.5 mM SSZ-treated cells, but at SSZ concentration of over 1.0 mM a dose-dependent prominent punctate patterns of blue fluorescence were observed, supporting autophagy induction by SSZ (Figure 4B).

#### SSZ enhanced the expressions of autophagy-related proteins

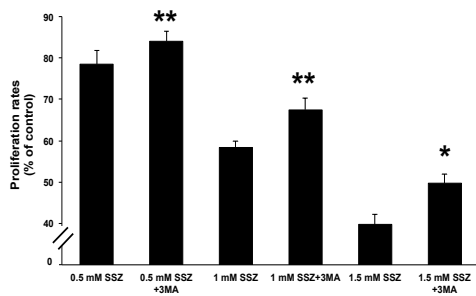
Western blotting was performed to confirm the induction of autophagy or apoptosis in SSZ-treated HSC-4 cells. As shown in Figure 5, dose-dependent increases in the conversion of LC3-I into LC3-II were observed in SSZ-treated cells. In addition, the expression of Atg5-12, a protein complex that plays a key role in the formation of autophagosomes, tended to increase after treatment with SSZ (Figure 5).



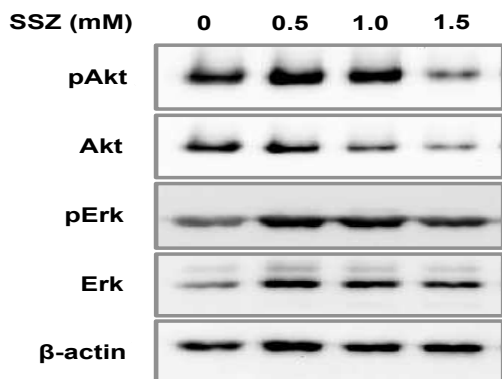
**Figure 3. Effects of SSZ on the Percentage of HSC-4 Cells Undergoing sub-G1 Arrest.** Percentages of HSC-4 cells undergoing sub-G1 arrest were determined after treating cells with the indicated concentrations of SSZ for 24 h. After SSZ treatment, propidium iodide staining was performed and percentages were determined by flow cytometry



**Figure 5. The expression of autophagy-related proteins in HSC-4 cells after treatment with the vehicle or 0.5, 1.0, or 1.5 mM SSZ for 24 h.** Western blotting was performed to detect LC3-I, LC3-II and Atg5-12 conjugated protein. Beta-actin was used as loading control



**Figure 6. Effects of SSZ on the Viabilities of HSC-4 Cells Pre-Treated with 3-MA.** HSC-4 cells were pretreated with or without 3-MA for 1 h and then with 0.5, 1.0, or 1.5 mM of SSZ for 24 h. Cell viability was analyzed using a MTT assay. Results are expressed as means±standard deviations. \*p<0.01, \*\*p<0.001 compared with non-pretreated cells treated with to same amount of SSZ



**Figure 7. Effects of SSZ on the Activations of Akt and ERK Signaling Pathways in HSC-4 Cells.** HSC-4 cells were treated with the indicated concentrations of SSZ and then Western blotting was carried out on lysates for Akt, phospho-Akt, ERK and phospho-ERK. Beta-actin was used as loading control

*SSZ treatment promoted autophagic cell death in HSC-4 cells*

To determine whether SSZ-induced autophagy is involved in cell survival or cell death, we performed an MTT assay on cells pre-treated with 3-MA (a classical inhibitor of autophagy) for 1 h prior to SSZ treatment. It was found that the cell viability of the 3-MA pre-treated and of SSZ-treated cells was significantly greater than that of SSZ treated-cells (Figure 6).

*The phosphorylation of Akt and ERK in HSC-4 cells in response to SSZ*

To explore the molecular mechanism of SSZ-induced

cell death, the expressions of phosphorylated Akt and ERK were examined. SSZ treatment suppressed the phosphorylation of Akt, but enhanced the phosphorylation of ERK (Figure 7).

**Discussion**

In this study, we investigated the possibility that SSZ has chemotherapeutic potential for the treatment of OSCC. Our results show that SSZ significantly inhibits the proliferation of HSC-4 cells. This finding is supported by positive AVO and MDC staining and the conversion of LC3-I to LC3-II, a hallmark of autophagy induction modulated by Atg5-12 conjugate (Ravikumar et al., 2009; Luo et al., 2012). Moreover, SSZ-induced autophagy was found to occur with the involvements of the AKT and ERK pathways. Furthermore, the anti-proliferative effect of SSZ was confirmed in several OSCC cell lines, namely, Ca9-22, HSC-2, HSC-3, SCC25, and YD10B cells (data not shown). These results suggest the possible use of SSZ for the treatment of OSCC.

Most research on the action modes of chemotherapeutic agents has focused on apoptosis, which is a major cell death pathway initiated by genotoxic stress. As a result, apoptosis-related molecules have been currently a major target of cancer therapies. However, autophagy induced by various chemotherapeutic agents can augment the anti-cancer potential of drugs (Pandey and Chandravati, 2012). The mode of autophagic action in cancer cells can be classified as follows. First, when some cancer cells possess defective autophagy-related genes, the replacement of these genes can induce cell death or inhibit cancer cell proliferation. Second, when cancer cells can potentially undergo protective autophagy, the suppression of autophagy or treatment with an autophagy inhibitor can induce apoptotic cell death. Third, cancer cells that undergo autophagic cell death offer a promising chemotherapeutic target. For these reasons, the manipulation of autophagy induction provides an effective anti-cancer strategy (Pandey and Chandravati, 2012; Zhou et al., 2012).

The presence of sub-G1 arrest indicates the presence of apoptotic DNA fragmentation and is considered hallmark of apoptosis (Shin et al., 2011). By contrast, recent studies have reported that G2/M or G1 arrest is related to autophagy (Yu et al., 2013). During autophagy, cell cycle inhibition usually enhances the proportion of cells in the G2/M phase without apparent increase of cells in subG1 phase (Newman et al., 2007). Given that the overall percentage of cells undergoing sub-G1 arrest in the present study was significantly lower than that of dead cell observed in the MTT assay, it would seem that apoptosis could not be principally responsible for reduction of HSC-4 cell viability induced by SSZ.

During the formation of autophagosomes, the carboxyl termini of LC3-I (Atg8 homologues) is cleaved by Atg4 homologues. As a results, LC3-I is converted to activated form, so-called LC3-II (Pandey and Chandravati, 2012). On the other hand, Atg12 is conjugated to Atg5 and then localized to autophagosomes. The Atg5-12 conjugate then promotes the conjugation of

LC3-II to the lipid phosphatidylethanolamine (PE) at the phagophore expanding autophagic membranes (Pandey and Chandravati, 2012). The LC3-II-PE conjugation, and related Atgs are believed to trigger vesicle expansion in a concerted manner by providing the driving force for membrane curvature (Pandey and Chandravati, 2012). After formation, autophagosome delivers cytoplasmic components to the lysosomes (autophagolysosome) then, the contents of the autophagosome are degraded via acidic lysosomal hydrolases.

Acridine orange can penetrate AVOs (a hallmark of autophagy) where it becomes protonated. MDC staining provides another means of assess autophagy induction as MDC labels mature autophagic vacuoles, that is, autophagolysosomes. In the present study, AVO and MDC punctate staining and the LC3-II/LC3-I ratio increased in SSZ-treated HSC-4 cells in a dose-dependent manner. Collectively, our results show that the HSC-4 cell death induced by low concentration of SSZ was caused by autophagy and but not by apoptosis. To the best of our knowledge, autophagy induction in OSCC cells by SSZ is a novel observation.

Autophagy can have two opposing effects, namely, cytoprotection and autophagic cell death. Autophagy was initially thought to act in a cytoprotective manner under starvation conditions and in the presence of pathologic infection and immunity (Luo et al., 2012). Under these circumstances, autophagosomes can be formed to degrade damaged microorganelles for energy production or to engulf and remove antigens (Zhou et al., 2012). However, when autophagy reaches a level that the cell cannot sustain, autophagic cell death can occur (Zhou et al., 2012). Thus, we investigated the specific role of SSZ-induced autophagy using 3-MA, classical autophagic inhibitor. 3-MA suppresses autophagy at the early stage of autophagosome formation by inhibiting the recruitment of class III PI3K to the membrane (Liu et al., 2011; Zhou et al., 2012). Furthermore, as 3-MA has been reported to be somewhat cytotoxic to HSC-4 cells (Ideo et al., 2009) its cytotoxicity has taken into account during analysis. Pretreatment of HSC-4 cells with 3-MA significantly diminished proliferation inhibition by SSZ in all experimental groups (Figure 5). Therefore, our results suggest that SSZ-induced autophagy leads to autophagic cell death, which suggests SSZ is of chemotherapeutic value.

One of the most important functions of Akt is associated with cell survival. It is well known that Akt suppresses both autophagy and apoptosis, in addition to phosphorylating Beclin 1 and BAD, which result in the inhibition of pro-apoptotic and pro-autophagic functions (Wang et al., 2012; Marino et al., 2014). Akt activation has been proposed to be a mechanism of autophagy suppression based on several observations (Degtyarev et al., 2008). First, activation of the Akt-mTORC1 axis can inhibit autophagy, for example, constitutively active Akt suppresses autophagy induction by rapamycin. Second, FoxO protein, which is downstream of Akt, and glucose metabolism can negatively regulate autophagy. Third, Akt inhibition is associated with increased mitochondrial superoxide and cellular reactive oxygen species (ROS)

levels (Degtyarev et al., 2008). Furthermore, Choe et al. (2012) and Degtyarev et al. (2009) suggested that the inhibition of Akt is associated with the induction of apoptosis and autophagy. Our finding that decreases in phospho-Akt levels are involved in the cell death elicited by SSZ in HSC-4 cells supports these previous findings.

The tumor suppressor Beclin 1 is a target of protein kinase Akt and essential autophagy related protein that is required for autophagosome initiation (Sun et al., 2011; Wang et al., 2012). Even though the relationships between MAPK pathways and autophagy are not well understood, it has been established that autophagy induction is inhibited by PI3K/Akt pathway but activated by the ERK pathway (Shin et al., 2011; Wang et al., 2012). In our results, Beclin 1 expression was not detected after SSZ treatment, as HSC-4 cells are Beclin 1-deficient.

The regulation of autophagy involves interaction between ERK and autophagolysosome formation (Shin et al., 2011). Wang et al. demonstrated that cisplatin-induced autophagy is regulated by the ERK pathway, which is in turn associated with the Atg5 pathway (Wang and Wu, 2014). In our results, treatment with SSZ induced activation of ERK pathway (Figure 7) and increased Atg5-12 complex (Figure 5) respectively. These results imply that SSZ may have molecular mechanisms similar as cisplatin. Considering the effects on Akt and ERK signaling pathways, these findings suggest SSZ-induced autophagy is regulated by Akt inhibition and ERK activation.

Our data indicate that pretreatment with 3-MA partially prevented the inhibition of cell proliferation caused by SSZ (Figure 5). The result suggests that SSZ-induced cell death is caused by another form of cell death, even after following for the cytotoxicity of 3-MA. On the other hand, at higher SSZ concentration, both cleaved caspase 3 (the effector caspase), and cleaved Poly (ADP-ribose) polymerase (PARP) (a caspase 3 substrate) levels were slightly increased (data not shown). Moreover, increased expression of LC3-II preceded that of cleaved caspase 3 and PARP (data not shown). Marino et al. previously described that anti-cancer agents can rapidly initiate autophagy and subsequently activate apoptosis (Marino et al., 2014). In addition, Lisiak et al. demonstrated that HIMOXOL induced autophagy and apoptosis in MDA-MB-231 cells (Lisiak et al., 2014). The present study shows that in HSC-4 cells, SSZ first triggers autophagy, which, in turn, induces apoptosis. Collectively, because SSZ induced cell death without apoptosis at low concentrations, our data suggested that autophagy is the major type of cell death induced by SSZ.

Taken together, the present study provides evidence that the Akt and ERK pathways are involved in SSZ-induced autophagic cell death in HSC-4 cells. To the best of our knowledge, this study is the first to document SSZ-induced autophagic cell death and its mechanism in OSCC cells. Furthermore, our findings suggest SSZ provides an attractive developmental route to novel therapeutics for the treatment of OSCC patients.

In conclusions, the present study demonstrates that SSZ can induce autophagic cell death in HSC-4 cells by inhibiting Akt and activating ERK signaling pathways.

These results indicated that SSZ has a potential as chemotherapeutic agent for the treatment of OSCC patients.

## Acknowledgements

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