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THE EFFECTS OF AUTOPHAGY AND SENESENCE ON SENSITIVITY TO CISPLATIN
IN HEAD AND NECK CANCER

by
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April 2020

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

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List of Abbreviations

3-MA: 3-methyladenine

ATG: autophagy related (protein)

Baf: bafilomycin A₁

Bcl-2/Bcl-xl: B-cell lymphoma 2, B-cell lymphoma extra large (proteins)

β-Gal: beta-galactosidase

C₁₂FDG: 5-dodecanoylaminofluorescin di-β-D-galactopyranoside

Cis: cisplatin

CQ: chloroquine

DMEM: Dulbecco's Modified Eagle Medium

HN12: cell line derived from head and neck lymph node metastatic tumor cells (p53-null)

HN30: p53 wild type cell line

HNSCC: head and neck squamous cell carcinoma

HPV: human papilloma virus

LC3: microtubule associated protein 1A/1B light chain 3B

mTOR: mammalian target of rapamycin

p62/SQSTM1: ubiquitin-binding protein p62/sequestosome 1

PBS: phosphate buffered saline

PI: propidium iodide

PI3K: phosphatidylinositol 3-kinase

SASP: senescence-associated secretory phenotype

TIS: therapy induced senescence

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

While current treatments in cancer, such as chemotherapy and radiation, can generally be effective in eliminating disease in patients, there also exists the possibility of recurrence of cancer cells over time. In patients diagnosed with locally advanced head and neck carcinoma, about 50-60% develop a loco-regional recurrence within two years, and 20-30% of patients develop metastatic disease at distant sites in the body [5]. On a cellular level, one mechanism for this survival may be that natural mechanisms such as autophagy and senescence play a role in allowing cells to survive after undergoing treatment. One standard of care chemotherapy for head and neck cancer is cisplatin, which was used as the primary treatment in this project. HN12 cells (head and neck tumor cells; p53-null) showed significant growth arrest and decreased viability in response to 5 μ M cisplatin treatment, but proliferative recovery over time. It was found that apoptosis did not play a significant role in this growth arrest, as assessed by annexin V/propidium iodide assays to measure apoptotic cell death. Additionally, cisplatin was shown to induce significant levels of autophagy in head and neck tumor cells with acridine orange staining and western blot analysis; however, pharmacological inhibition of autophagy with either chloroquine (5 μ M), bafilomycin (5 nM), or 3-methyladenine (1 mM) did not sensitize cells to treatment with cisplatin, indicating a nonprotective role of autophagy. Additionally, HN12 cells showed significant levels of senescence, a form of cellular growth arrest, as indicated by beta-galactosidase upregulation, flattened morphology, and cell cycle arrest. Targeting of senescent cells with the senolytic ABT-263, a Bcl-2/Bcl-xl inhibitor, was effective in sensitizing cells to treatment with cisplatin, but cellular proliferation still occurred over time. Additionally, the HN30 cell line (p53 wild type) was compared to the HN12 line and experiments suggested that

p53 status did not play a significant role in induction of autophagy or the sensitization to the senolytic, although more work needs to be done. Overall, these studies provide evidence that autophagy is nonprotective in the HN12 cell line in response to cisplatin treatment, and that senescence plays a role in allowing cells to survive over time. Further, ABT-263 is effective in targeting the remaining population of senescent cells after cisplatin treatment and may be a potential therapeutic mechanism in preventing the recurrence of head and neck cancers.

Introduction

HEAD AND NECK CANCER

Head and neck cancers are caused by abnormal and malignant growth of squamous cells that line the mucosal surfaces inside the head and neck regions. These regions include the oral cavity, which encompasses the tongue, lips, gums, inside of the cheek, and the hard palate; they also include the pharynx (includes the throat, soft palate, base of the tongue, and tonsils), larynx, paranasal sinuses, and even the salivary glands, although this form of cancer is more rare. Head and neck squamous cell carcinomas (HNSCC) are the sixth most common cancer in the world [14]. Head and neck cancers account for about 4% of all cancer cases in the United States [1] and about 550,000 cases globally, per year [2, 3]. Further, about twice as many men as women are diagnosed with head and neck cancer, and it is more prevalent in those aged 50 and older according to the Center for Disease Control and Prevention. The new numbers of cases of oral cavity and pharynx cancers from 1999 to 2016 has steadily increased through the years most likely due to the growing population in the country [3]. In 2016 in the United States, over 45,000 cases of oral cavity and pharynx cancer were reported, resulting in over 10,000 deaths [3]. These statistics point to the importance of developing more targeted and effective treatments for patients diagnosed with head and neck cancers.

The main causes of head and neck cancer vary depending on country, environment, and past viral exposure; however, one of the most common causes is alcohol and tobacco use which accounts for approximately 75% of all head and neck cancer cases. It has been reported that

despite decrease in smoking and tobacco use since the 1980's, some countries such as the United States, Canada, and the UK have seen an increase in cases of oropharyngeal and oral cavity cancers [2]. Another prominent risk factor is sexually transmitted infection with the human papilloma virus (HPV), in particular types 16 and 18, which is linked to approximately 70% of cancers in the oropharynx and for which the correlation with incidences of oropharyngeal cancers in the US is growing [3]. Further, tumors can even be classified as HPV-positive or HPV-negative, and the incidence of HPV-positive head and neck cancers are increasing; however, p53 mutations are more frequent in HPV-negative cancers [15]. Other causes include poor oral health, chewing of betel quid (most common in South Asian regions), occupational exposures to various substances such as asbestos and wood dust, radiation exposure, and prior infection with the Epstein-Barr virus, which is a risk factor for nasopharyngeal and salivary gland cancers. One factor to keep in mind when considering prominent risk factors is the environment; alcohol and tobacco use are more likely risk factors for those in western countries whereas betel nut chewing is a common risk factor in south Asian regions [14].

Survival and prognosis following diagnosis with head and neck cancers varies based on tumor size, location, metastases, and even HPV status. Those with early stage cancers (about one-third of patients) usually have favorable outcomes after surgery or treatment. More often, however, patients are diagnosed with advanced or metastatic cancer which must be targeted with chemotherapy and radiation; importantly, treatments for these advanced cancers have not improved significantly over the past 30 years [15]. Disease can progress by metastasizing at distant locations or second primary tumors can develop [15].

This project mainly utilizes the HN12 head and neck tumor cell line, which is derived from head and neck lymph node metastatic tumor cells. This cell line is a p53-null cell line, which allows us to potentially study the role of this gene in the advancement of these cancers. Specifically, this cell line is p53-truncated and has a mutation in the exon 7 splicing donor site of the gene [40]. p53 has been found to be one of the most commonly mutated genes in head and neck cancers, [14] and p53 mutations are one of the most frequent occurrences in human cancer. Data from the HN30 cell line (p53 wild type), generated by Fereshteh Ahmadinejad from our lab, are used as a comparison to the p53-null cells to further elucidate its potential role in this process.

CURRENT TREATMENTS

Treatment for head and neck cancers vary and depend on multiple factors including the person's health, location of the tumor, tumor accessibility, and stage of disease. Current treatment options can include surgery to remove the tumor, chemotherapy, radiation, immunotherapy, or a combination of therapies. The standard of care for chemotherapy treatment for head and neck cancers is three-week cycles of 100 mg/m² (5-10 uM) intravenous cisplatin administration with concurrent radiation [2]. Other uses for cisplatin include chemotherapy for bladder cancer, ovarian cancer, testicular cancer, lung cancer, breast cancer, stomach cancer, and prostate cancer.

Cisplatin (cis-[Pt(NH₃)₂Cl₂]; *cis*-diamminedichloroplatinum) is a square planar platinating agent; the drug binds to the DNA backbone and cross links purine bases, creating

intra-strand and inter-strand linkages [13]. The formation of these cross links in DNA can inhibit transcription factors from accessing the DNA, which prevents DNA synthesis, repair, and RNA transcription. Cisplatin can also induce mispairing of nucleotides, leading to cell death, or apoptosis. One common combination therapy used for head and neck cancers is use of docetaxel (Taxotere), cisplatin (Platinol), and fluorouracil (TPF) followed by radiation therapy, which shows improvement in quality of life [10]. Docetaxel works by preventing microtubule disassembly [11] and fluorouracil replaces the nucleotide uracil, preventing RNA transcription [12]; these processes lead to tumor cell death.

Toxicity resulting from administration of cisplatin can be severe and dose-limiting. The main side effects associated with cisplatin are nausea and vomiting, renal toxicity, immune suppression, allergic reaction, ototoxicity and hearing loss, and peripheral neuropathy. Clearly these side effects are serious and can be a cause for concern in many patients. One study published in 2017 found that cisplatin is retained inside the cochlea for years after treatment, and that 40-80% of patients suffer permanent hearing loss [8]. According to another study analyzing the mechanisms of nephrotoxicity in patients receiving cisplatin treatment, kidney damage is one of the main dose-limiting effects of the drug, and 20-30% of patients develop acute kidney injury (AKI). Regardless of these side effects, cisplatin is still widely prescribed [9]. Other drugs used to avoid negative side effects are carboplatin, and oxaliplatin, which are also platinum-containing agents and may have differing side effects and potencies [13]. Other more targeted drugs are also used in combination with chemotherapy or radiation including the epidermal growth factor receptor (EGFR)-specific antibody, cetuximab [15].

RESISTANCE AND RECURRENCE

One consistent challenge in the struggle to eliminate cancer in patients is resistance to treatment and eventual recurrence of the disease. Metastasis may occur when cancer spreads to areas of the body other than the initial site of the cancer. Metastasis of HNSCC occur through spread in lymph nodes in the neck [15]. In response to cellular stresses such as chemotherapy and radiation, cells can respond in a variety of different ways, potentially as self-survival mechanisms (see figure 4).

One of the most studied cellular response to chemotherapy is apoptosis, which is characterized by rounding up of the cell, reduction of cellular volume, plasma membrane blebbing, and DNA condensation and fragmentation. Caspases are proteins that initiate this process of cell death. Cells shrink, the membrane begins to bulge and bleb, and then the cell breaks into apoptotic bodies which are then phagocytosed. This process can be induced by a variety of factors including DNA damage, oncogene activation, and oxidative stress. Several proteins regulate this process; of importance are Bax and Bak which are “pro-apoptotic” proteins, and Bcl-2 and Bcl-xl, which are anti-apoptotic proteins and promote cell survival over apoptosis.

In patients, many cancer cells die via apoptosis in response to treatment with drugs like cisplatin, but in some cases, populations of cells can survive, potentially leading to recurrence of cell growth and metastasis. Processes such as autophagy and senescence can contribute to this survival.

AUTOPHAGY

One of these potential cancer cell survival mechanisms is macro-autophagy (referred to here as autophagy), or “self-eating,” which can occur during times of stress or nutrient deprivation. This is a dynamic process in which cellular contents are incorporated into autophagosomes, which fuse with lysosomes and are then degraded to generate energy and metabolic precursors (see Figure 1). It is essentially a natural mechanism cells undergo in order to maintain homeostasis by degrading damaged organelles and cellular products to reutilize for energy. This process is initiated by a phagophore, or a lipid bilayer isolated membrane. This phagophore then undergoes an elongation step in which it expands to engulf cargo such as proteins and organelles and sequesters them into a double membrane autophagosome. In the final step, the autophagosome matures and then docks and fuses with an acidic lysosome, forming an autolysosome, which promotes degradation by lysosomal hydrolases. The degraded products can then be reutilized by the cell.

Various proteins play a role in this complex and intricate process. Of importance to this project are markers such as LC3B and p62/SQSTM1. LC3 (Microtubule associated protein 1A/1B light chain 3B) is a widely used marker for autophagosomes. Upon autophagy induction, (cytosolic) LC3-I is cleaved and conjugated by phosphatidylethanolamine (PE) to form LC3B-II; this is then recruited to the inner and outer surfaces of autophagosomes and remains bound when the membrane is fused to a lysosome. LC3-II is degraded along with the other cellular contents at the completion of autophagy [16]. p62/SQSTM1 is another protein that binds to LC3, which promotes its uptake and degradation by the process of autophagy. Inhibition of the *later* stages of

autophagy via pharmacological inhibitors such as chloroquine or bafilomycin A₁ may result in accumulation of these proteins since they block fusion of the autophagosome to the lysosome, resulting in accumulation of autophagosomes (see figures 2 and 3). Inhibition of *earlier* stages of autophagy by 3-methyladenine, for example, or by genetic silencing of autophagy regulatory proteins such as ATG5 or ATG7, would result in the inability to form autophagosomes, causing no LC3I to LC3II conversion, and a buildup of cytosolic LC3-I instead (see figure 3).

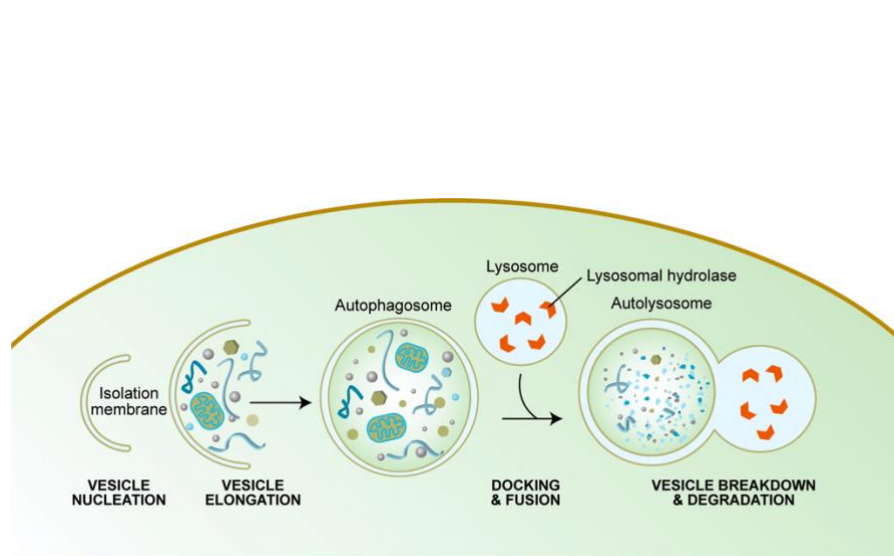


Figure 1. Schematic diagram of the steps of autophagy

Meléndez, A. and Levine, B. Autophagy in *C. elegans* (August 24, 2009), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.147.1, <http://www.wormbook.org>.

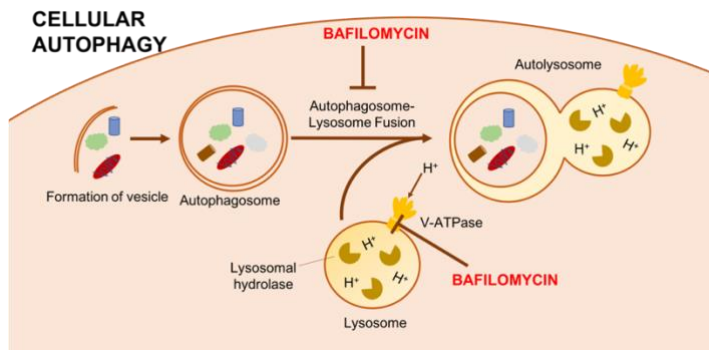


Figure 2. Mechanisms of bafilomycin A1 autophagy inhibition.

Wikipedia contributors. (2020, April 14). Bafilomycin. In *Wikipedia, The Free Encyclopedia*

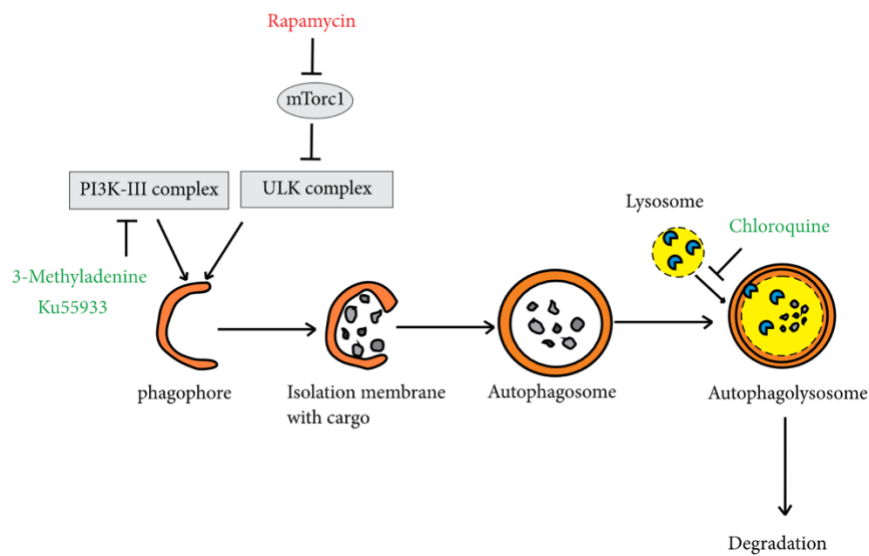


Figure 3. Mechanisms of autophagy inhibition by chloroquine and 3-methyladenine

Bagamanshina, A. V. *et al.* (2019). Cytotoxic and Antitumor Activity of Lactaplin in Combination with Autophagy Inducers and Inhibitors. *Hindawi BioMed Research International*.

Autophagy can play dual roles in cancer. One possibility in response to treatment with chemotherapy is tumor suppression; autophagy could play a role in autophagic cell death and prevent aggregation of damaged cellular products, or it could play a role in tumor growth by allowing the cell to survive by self-eating to generate energy. Autophagy in fact has been shown to have up to four distinct forms [6]. The most commonly thought of form in response to chemotherapy is protective autophagy; studies have shown that pharmacological inhibition of this form of autophagy can have a therapeutic effect in the clinic. For example, one study showed a cytoprotective role with cisplatin treatment in renal proximal tubular cells, and the use of autophagy inhibitors sensitized cells and increased apoptosis [19]. Autophagy can also show cytotoxic functions; cytotoxic autophagy is associated with reduction of viable cells and when this process is inhibited, cells become less sensitive to treatment. Cytostatic autophagy inhibits cellular growth, and inhibition of this protects the cell from treatment, similar to cytotoxic autophagy [6]. Nonprotective autophagy may occur when cells display degradative functions, but inhibition of this form of autophagy does not have an impact on sensitivity to chemotherapy. This may be important in the clinic, where it is critical to understand whether a certain drug elicits a protective or nonprotective form of autophagy in a certain cancer before administering an additional drug to a patient [20]. Further, there is evidence of a process termed the “autophagic switch,” in response to chemotherapy. One recent study from our lab evaluated the response to cisplatin in non-small cell lung cancer cells that were either wild-type, or p53-null; inhibition of autophagy induced nonprotective autophagy in the p53 wild-type cells, while p53-null cells showed cytoprotective autophagy [18]. Therefore, it cannot be assumed that chemotherapy treatment will always induce a cytoprotective response and that drugs such as chloroquine or hydrochloroquine (the only FDA approved autophagy inhibitors available) will

always enhance cellular sensitivity to treatment [20]. Further, stress induced autophagy may exhibit different forms in different cell lines or with different treatments [20]. In our efforts to sensitize cancer cells to treatment in the clinic, it is important to elucidate and further study these forms of autophagy because the functional form could or could not have an influence on drug sensitivity.

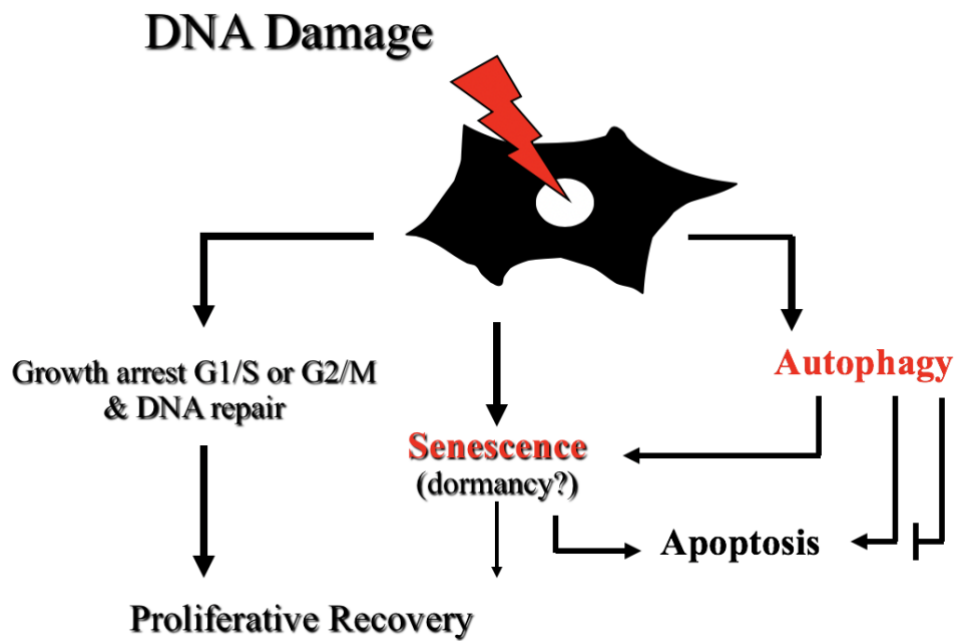


Figure 4. Effects of DNA Damage in the cell
Dr. David Gewirtz, Virginia Commonwealth University

SENESCENCE

Senescence is another known response to cellular stressors and DNA damage and can be, as some research is beginning to show, a mechanism of cell survival. Senescence is a normal process in aging and can be defined as a process in which cells are growth arrested and fail to divide but are still metabolically active. These cells are resistant to apoptosis and secrete a variety of chemokines and cytokines (including IL-6 and IL-8, mediators of inflammation), called the “senescence associated secretory phenotype (SASP).” Forms of senescence include replicative senescence, oncogene induced senescence, and stress-induced senescence. Replicative senescence occurs when telomeres (the repeated complex at the end of DNA, TTAGGG, which protect the chromosomes from degradation) are lost with each cell division and enter into a growth arrest. Oncogene-induced senescence is caused by either a mutation of an oncogene or the inactivation of a tumor-suppressor gene [21]. The final and most relevant form to this paper is stress-induced senescence caused by factors such as oxidative damage, DNA damage, radiation, and chemotherapy.

Various morphological markers are observed in senescent cells including cellular enlargement and flattening, increase in the size of the nucleus, and appearance of cytoplasmic vacuoles. One key feature important to this project include the expression of beta-galactosidase, which is a lysosomal enzyme upregulated in senescent cells; it is only active at a pH of 6, whereas the normal lysosomal pH is 1-2. Senescent cells have also been shown to induce p21 and p16, prominent cyclin-dependent kinase (CDK) inhibitors, which may allow the cell to survive [22].

This therapy induced senescence (TIS) has previously been thought of as a favorable and irreversible response to chemotherapy, but new research suggests that tumor cells can escape this form of senescence and that this may lead to disease recurrence [23]. Studies have shown, for example, that although Doxorubicin induces senescence in MCF-7 breast tumor cells, a small population was able to escape this growth arrest and contribute to resistance [26]. Senolytics are agents that specifically target senescent cells and include drugs such as histone deacetylase (HDAC) inhibitors and Bcl2 (anti-apoptotic protein) inhibitors like ABT-263 [24]. A study looking at the effect of radiation on pulmonary fibrosis found that ABT-263 killed senescent pneumocytes and reversed pulmonary fibrosis, pointing to the significance of senescence in disease processes [25].

HYPOTHESIS

Autophagy and senescence are known to occur as a response to chemotherapy in various cancer cell lines, suggesting that both responses serve to protect the cell from external stressors and damage. The question to be answered, however, is what might prove to be the most effective way to sensitize these cancer cells to treatment in order to prevent proliferation and recurrence. Various studies have shown that cisplatin induces a protective form of autophagy; if this is the case, it may indicate a promising target in sensitizing cells to treatment. Other studies have also shown that cells undergo senescence in order to evade death, suggesting another potential therapeutic target.

Methods

Cell Culture and Treatment

HN12 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific), 100 u/ml penicillin G sodium (Invitrogen), and 100 ug/ml streptomycin sulfate (Invitrogen). Cells were incubated at 37° C under humidified 5% CO₂. Media was replaced every other day.

Cell Lines

HN12 cells were obtained from the upper aerodigestive tract of head and neck carcinomas. Cells were derived from lymph node metastatic lesions. HN30 cell line was obtained from the lab of Dr. Hisashi Harada, Virginia Commonwealth University.

Trypan Blue Exclusion for Cell Viability

Temporal studies of cell growth inhibition were based on cell viability by trypan blue exclusion. Cells were stained with 0.4% trypan blue (Sigma-Aldrich) and counted using a hemocytometer. Cells were trypsinized and resuspended; 10 µL were loaded into the hemocytometer and counted under a microscope.

Clonogenic Survival

Cells were plated in a density of 500 cells per dish and treated with the respective drug condition for 24 hours. After drug treatment, media was replaced every other day. Cells were allowed to grow and visible colonies in all conditions had formed by Day 18 after drug treatment. After

colonies formed during incubation, cells were washed in phosphate buffered saline (PBS; Life Technologies, Grand Island, NY) and then fixed with 100% methanol. Colonies were then stained with 0.1% crystal violet dye (Sigma-Aldrich) and counted. Data is either expressed as percentage of colonies as compared to controls, or as total colony counts (these are indicated in respective figure legends).

Annexin V/Propidium Iodine Assay for Apoptosis

Cells were plated and drug treated on the following day for 24 hours. On Day 3 after drug treatment, cells were collected and apoptosis was measured by annexin V-FITC/propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences) according to the manufacturer's instructions. Samples were analyzed by BD FACSCanto II and BD FACSDiva software at Virginia Commonwealth University Flow Cytometry Core Facility.

Western Blot Analysis

Western blotting was performed as described previously [39]. Analyses on western blots were performed using Image J2 software. Blots were incubated with primary antibody (1:1000) overnight, washed, and then tagged with secondary antibody (anti-rabbit, 1:2000) for 2 hours at room temperature. The primary antibodies used are as follows: LC3B (Cell Signaling Technology), and GAPDH (Cell Signaling Technology Inc., Danvers, MA). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, anti-rabbit).

Acridine Orange Autophagy Detection

Cells were seeded in 6-well plates and treated with cisplatin or autophagy inhibitors for 24 hours on the next day. On day 3 after drug treatment, cells were stained with 1 $\mu\text{g/ml}$ acridine orange at 37° C for 20 minutes and then washed with Phosphate Buffered Saline. Cells were observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan). For quantification of autophagic vesicles (AVOs), cells were trypsinized after stain removal, harvested, and washed with PBS. Pellet fractions were resuspended in PBS and analyzed by BD FACSCanto II and BD FACSDiva software. All experimental procedures were performed with cells protected from light.

Beta-Galactosidase Senescence Detection and C₁₂FDG Staining

Beta-galactosidase (β -gal) staining was performed to detect senescence. Cells were incubated for one hour with 100 nM Bafilomycin to achieve lysosomal alkalization, followed by staining with C₁₂FDG (10 μM , Thermo Fisher Scientific) for 2 hours at 37° C. After incubation cells were trypsinized, collected, and washed with PBS. Pellet fractions were resuspended in PBS and analyzed by flow cytometry.

X-Gal Staining

Cells were seeded in 60 mm plates and treated with cisplatin for 24 hours on the next day. On each respective day after drug treatment during the time course, cells were washed with Phosphate Buffered Saline then fixed at room temperature for 5 minutes. Fixing solution was made using 2% formaldehyde and 0.2% glutaraldehyde. Cells were then washed in PBS again and then stained with X-Gal. The staining solution consisted of 20 mg/ml X-gal in

dimethylformamide, 0.2 M citric acid/Na phosphate buffer (pH 6.0), 100 mM potassium ferrocyanide, 100 mM potassium ferricyanide, 5 M sodium chloride, 1 M magnesium chloride, and deionized water. Cells were then incubated at 37° C in a CO₂-free incubator to preserve the pH of the solution. Staining solution was then removed, cells were washed in PBS, and then imaged.

Cell Cycle Analysis

Cells were plated and drug treated for 24 hours on the following day. Cells were trypsinized and collected on each respective day as pellets. Pellets were washed in PBS then resuspended and stored in 70% ethanol at -20° C before being analyzed by flow cytometry.

Statistical Analysis

Data were expressed as mean \pm SD from three separate experiments. One-way ANOVA followed by Bonferroni's post-hoc test was used to assess statistical differences between groups. Levels of significance were assessed using GraphPad Prism 8.0 software. A p-value of <0.05 was considered statistically significant. Data were collected from three independent experiments, unless indicated otherwise. "n" indicates the number of total experimental repeats. Each experimental repeat was performed in three separate wells per each condition, and data is represented as a mean of these three triplicates.

Results

1. Cisplatin dose-dependently inhibits cell growth

An initial analysis of the effects of cisplatin concentration on HN12 cell viability was necessary to gauge how cells responded to treatment. Therefore, a time course was used to assess the cytotoxic and cytostatic effects of cisplatin on HN12 cells. Cell viability was assessed using a Trypan blue exclusion assay, as described in the methods, and viable cells were counted on each respective day. Cells were treated with either 2, 5, or 10 μM cisplatin for 24 hours. The data show that cisplatin concentration-dependently inhibits cell growth over time, but that cells begin to show proliferative recovery around day 8. Up to around 6-8 days after treatment with cisplatin, cells are significantly growth arrested, indicating a strong response to treatment. After assessing these results, a 5 μM concentration of cisplatin was chosen to perform the rest of the assays, as 10 μM was too toxic a dose to assess cell viability and response to treatment.

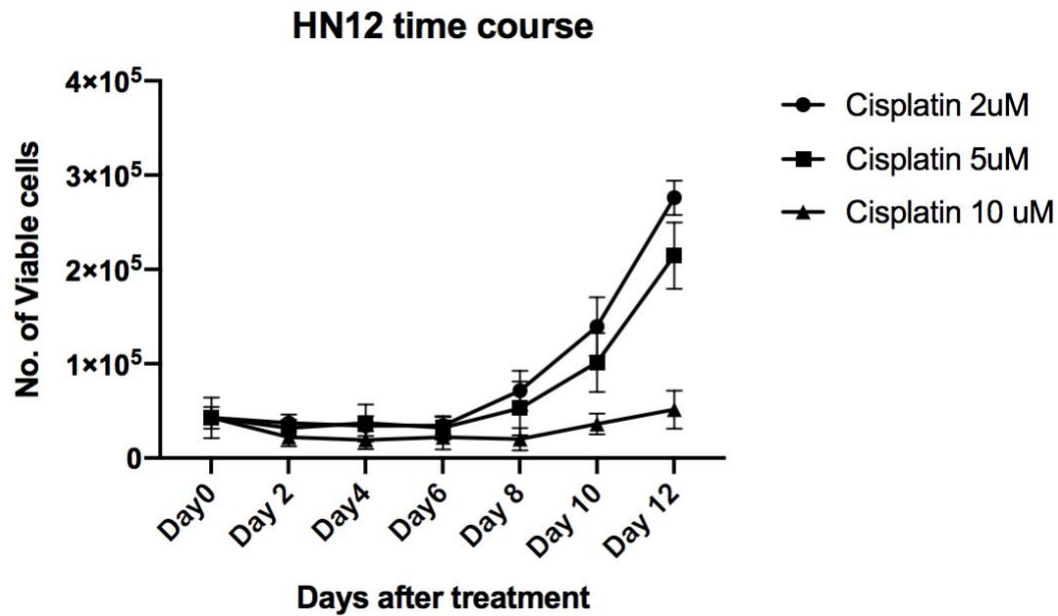


Figure 1. Effect of cisplatin concentration on cell viability over time. HN12 cells were treated with either 2, 5, or 10 μM cisplatin for 24 hours and then drug-free media was replaced every other day. On each respective day viable cells were counted by a hemocytometer (n=3). Data generated by Fereshteh Ahmadinejad.

2. Role of apoptosis in cell growth inhibition

In order to assess whether apoptosis is playing a role in the sensitivity of HN12 cells to cisplatin, an Annexin V/Propidium Iodine assay was performed to assess the percentage of apoptotic cells present after drug treatment. It was found that apoptosis does not play a significant role in sensitivity to the drug; although cisplatin induces some cell death, the extent is minor and occurs at a maximum level of about 20%. The previous assay of cell viability in response to cisplatin showed a significant reduction in cell growth, indicating that some other process such as autophagy or senescence may be responsible for this observation.

Additionally, cisplatin induced a statistically insignificant amount of apoptosis compared to controls in both the HN12 (p53 null) and HN30 (p53 wild type) cell lines. This indicates that both cells with and without functional p53 undergo apoptosis to a similar extent. Both cell lines after undergoing treatment with cisplatin induce a similar amount of apoptosis, suggesting that p53 does not play a role in this specific stress response in response to this treatment. As a reference, the IC₅₀ values had been previously determined to be approximately 40 μ M in the HN12 cell line and 15 μ M in the HN30 cell line.

In future studies, it would be beneficial to examine the level of apoptosis over time by conducting a time course and measuring the percentage of apoptosis on each day; for example, 24 hours after drug treatment and various time points up to seven days. Additionally, a dose-response assay would be useful in determining which concentrations of cisplatin induce the most apoptosis; for example, concentrations of 1, 2.5, 5, and 10 μ M cisplatin.

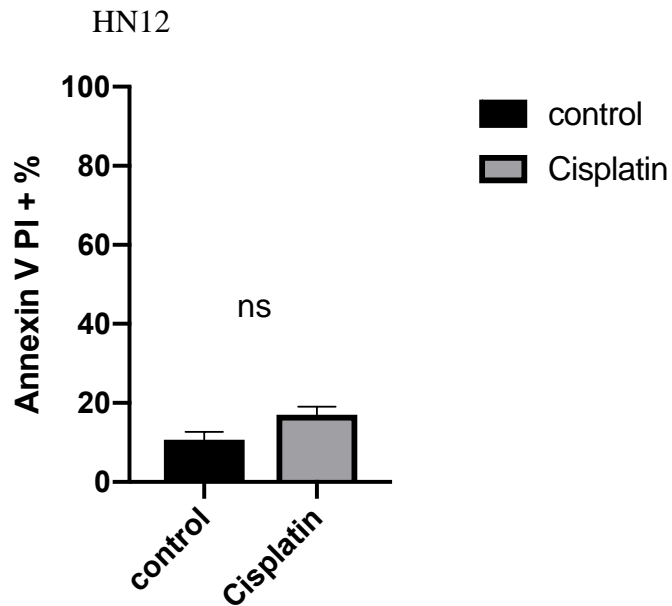


Figure 2A

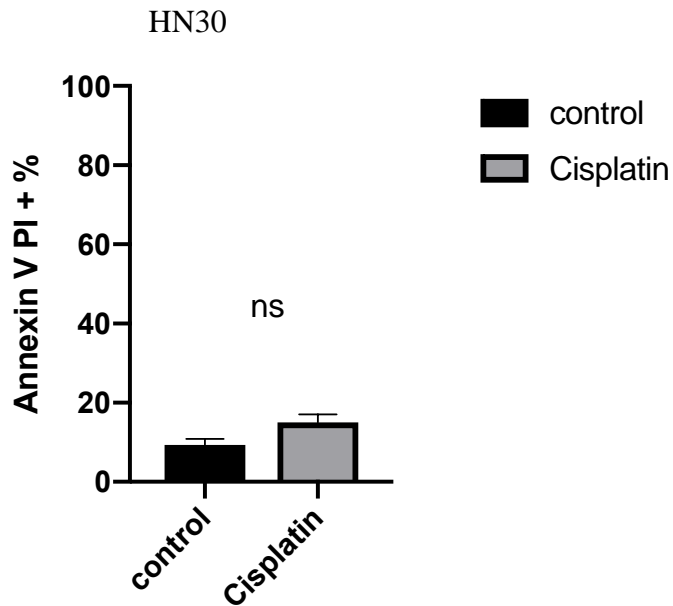


Figure 2B

Figure 2. Effect of cisplatin on the percentage of apoptotic cells as compared to basal levels. Flow cytometry was used to detect the level of FITC-Annexin and Propidium Iodide dye present in each cell. Total apoptosis was calculated by adding percentages of early and late apoptosis. Cells were treated with 5 μ M cisplatin for 24 hours and were collected on day 5 after treatment for flow cytometry. IC₅₀ values were determined to be approximately μ M in the HN12 cell line and 15 μ M in the HN30 cell line. A) Levels of apoptosis in the HN12 (p53 null) cell line. B) Levels of apoptosis in the HN30 (p53 wild type) cell line. (n=3); *p<0.05 as compared to untreated controls. Data generated by Fereshteh Ahmadinejad.

3. Cisplatin induces autophagy over time and dose-dependently

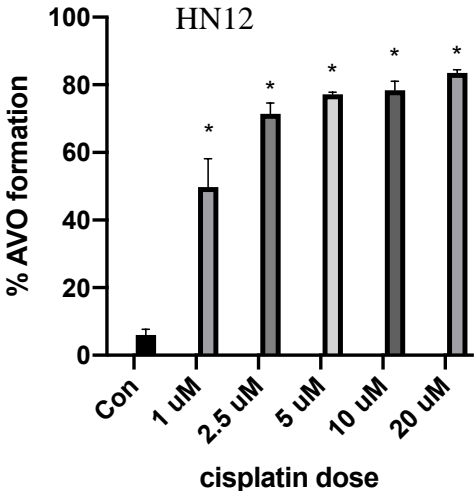
Since cells did not show significant levels of apoptosis, it was predicted that autophagy could potentially be playing a role in this process as a pro-survival mechanism. To assess the role of autophagy in cisplatin-treated cells, the Acridine Orange staining assay was used. As discussed in the methods, acridine orange is a cation dye that fluoresces an orange-yellow color in the presence of a low pH environment and is therefore used to as an initial screen to detect the induction of autophagy as it accumulates in acidic autophagic vesicles. Acridine orange fluorescence can be quantified using flow cytometry and visualized using live cell imaging.

As shown in Figure 3A, cisplatin dose-dependently induces autophagy and reaches a maximum level of autophagy with higher concentrations that are significantly different from the control population. Approximately 80% of cells show autophagy induction with 5 μ M of cisplatin treatment as compared to basal levels. In figure 3B obtained from live cell imaging, treatment with 5 μ M cisplatin induces autophagic vesicles which persist over time, and up to five days after removal of the drug. Autophagic vesicles are seen as soon as 24 hours after removal of cisplatin in both HN12 (p53 null) and HN30 (p53 wild type) cell lines. This indicates that p53 status does not necessarily play a role in the induction of autophagy by cisplatin. However, the nature of autophagy needs to be further assessed in both cell lines.

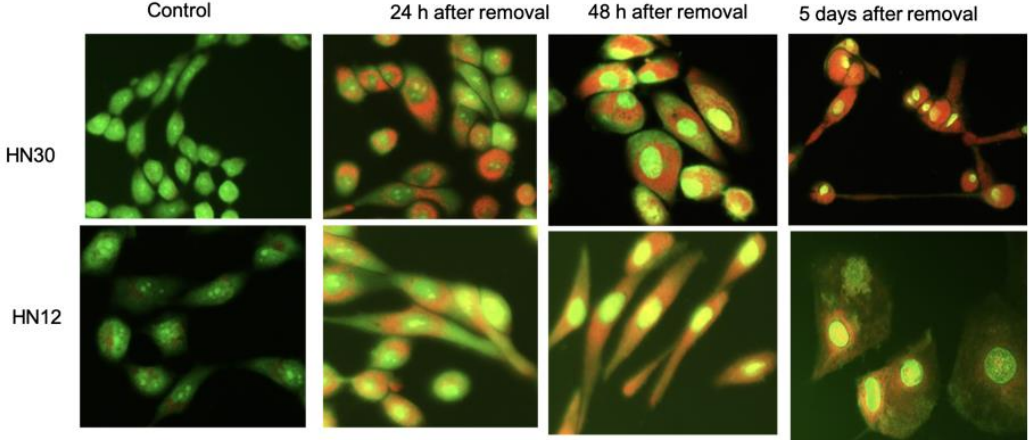
Additionally, western blot analysis after treatment with cisplatin for 24 hours indicates increased levels of LC3 over time, suggesting the induction of autophagy which persists up to 5 days after drug removal. These results indicate that cisplatin induces a significant level of

autophagy, and to a greater extent than the induction of apoptosis caused by the same concentration of cisplatin.

3A.



3B.



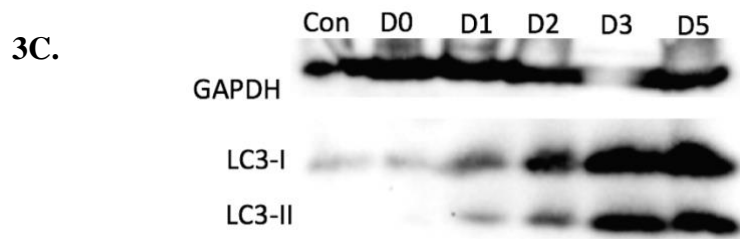


Figure 3. Cisplatin induced autophagy in HN12 cells. A) Effect of concentration of cisplatin on induction of autophagic vesicles. Cells were treated with either 1, 2.5, 5, 10, or 20 μM of cisplatin for 24 hours. Cells were harvested and collected for flow cytometry on Day 3 after drug removal. (n=3); * $p < 0.05$ as compared to controls B) Effect of cisplatin on induction of autophagic vesicles over time. Cells were treated with 5 μM cisplatin and drug was removed after 24 hours, and then replaced with drug-free media every other day until the day of imaging. Data generated by Fereshteh Ahmadinejad. C) Effect of 5 μM cisplatin on levels of LC3B over time (n=2). Cells were treated for 24 hours then collected on each respective day. D0 (Day 0) indicates 24 hours after drug removal.

4. Effects of inhibition of autophagy

In order to elucidate the functional form of autophagy induced in response to cisplatin exposure, pharmacological inhibitors of late-stage autophagy, chloroquine (CQ, 5 μ M) or bafilomycin A₁ (Baf, 5 nM) were utilized. Chloroquine is a drug that prevents lysosomal acidification and prevents fusion of the autophagosome to the lysosome (prevents completion of autophagy), and therefore results in accumulation of autophagosomes. Bafilomycin A₁ also prevents fusion of autophagosome to lysosome by acting as an ATPase inhibitor, preventing the acidification of the lysosome. 3-methyladenine is different in that it inhibits autophagy at an earlier stage by blocking formation of the autophagosome.

In the live cell images (Figure 4A), cells were treated with cisplatin for 24 hours and stained with acridine orange 48 hours post-treatment. The control panel shows a basal level of autophagy occurring with minimal orange puncta. In the cisplatin treated group, the cells show increased orange puncta, increased size, and increased acidic vesicle staining, indicating an increased level of autophagy. In the cells treated with cisplatin in combination with either CQ or Baf, inhibition of lysosome acidification is occurring, as indicated by the presence of yellow staining which occurs as a result of the increase in pH. Since CQ and Baf are late-stage autophagy inhibitors that prevent autolysosome formation, there is an increased presence of autophagosomes. The enlarged and flattened appearance of these cells is intriguing as all images are presented at the same scale; this change in morphology seen here is a traditional marker of senescence and prompted the investigation of senescence as presented in the next section. This may also be evidence of autophagy and senescence occurring in the same cell. One limitation of this assay is

that it is a rough estimate of the extent of autophagy; cells treated with CQ alone did not show any significant increase in orange staining, therefore autophagy inhibition needed to be confirmed via western blot analysis.

The live cell imaging was followed up with a western blot analysis to show LC3 accumulation as autophagy is inhibited (Figure 4B). The results indicated that autophagy was successfully inhibited in CQ, Baf, Cis + CQ, and Cis + Baf conditions. Normal LC3 conversion occurred in the cisplatin-treated condition, indicating sufficient autophagy induction and completion. In the conditions treated with CQ and Baf, there is increased LC3-I and LC3-II, compared to controls and cisplatin-treated groups, indicating late stage autophagy blockade resulting in autophagosome build up. In order to strengthen the conclusion that autophagy is adequately inhibited, it would be beneficial to also examine the effect of cisplatin treatment on p62/SQSTM1 degradation as a measure of autophagy proceeding to completion and its subsequent accumulation during blockade.

Additionally, a time course experiment conducted over 14 days showed a significant decrease in cell viability after treatment with cisplatin (Figure 4C); treatment with cisplatin in combination with chloroquine showed a similar trend. Treatment with 5 nM bafilomycin A₁ (Figure 4D) had an effect in sensitizing cells to treatment; however, the effect here may be additive rather than true sensitization by autophagy inhibition due to the similar percentage of decreased cell viability when comparing Baf and Cis + Baf conditions. As autophagy inhibition had no significant effect on increasing or decreasing cell viability, these results indicate that

autophagy does not play a major role in sensitivity to cisplatin and suggests that the role of autophagy in HN12 cells shown here is *nonprotective*.

One potential reason for the difference in the strength of Baf and CQ in their ability to sensitize cells could be their respective mechanisms of action. Although both alter lysosomal pH, CQ mainly impairs autolysosome fusion, and Baf A₁ inhibits the acidification of the lysosome by inhibiting ATPases and therefore its ability to degrade cellular components; therefore, the two drugs are not interchangeable [27]. One likely effect is that because there are ATPases throughout the cell, Bafilomycin has off target effects which increase toxicity. Also, it was found by Mauthe et. al. that Baf increased LC3-II levels more than treatment with CQ in U2OS (osteosarcoma) cells [27]. They also found morphological differences: treatment with Baf caused the cells to have intact cytoplasm in the lysosome (due to loss of degradation capacity), and CQ treated cells did not. From this observation it seems that altering the lysosomal pH by Baf may have an impact on the cell in other off-target mechanisms (perhaps inability to degrade waste and toxins and or lysosomal functions), while CQ is more specific to the autophagic process and formation of the autolysosome. The main difference may be that treatment with CQ does not lead to nonfunctional autolysosomes, whereas Baf does.

Pharmacological inhibition of early stage autophagy was also assessed using 1mM 3-methyladenine (Figure 4E) over 18 days. Cisplatin significantly reduced cell viability and induced growth arrest for up to 10 days; after this, cells begin to recover. The effect of autophagy inhibition in this case may also be nonprotective since there is a similar trend showing no effect on cisplatin sensitivity.

Figure 4F shows a clonogenic assay, another technique to assess survival of cell colonies after 24 hours of drug treatment. The results show that cells treated with cisplatin, either alone or in combination with autophagy inhibitors, showed significant decrease in colony formation and cell viability. There was no significant difference between these groups, however. A limitation of this assay is that it does not distinguish the cause of the growth arrest in these cells, but it is still useful to evaluate sensitivity to the drug. Further, in all repetitions of this experiment there was a floor effect and the conditions treated with cisplatin showed a decrease in colony formation such that we are not able to accurately distinguish the difference between the lower values. Therefore, this assessment cannot firmly show the form of autophagy taking place and no solid conclusions can be made. This assay would be a better tool to assess cytotoxic autophagy which would be indicated by an increase in cell viability when autophagy is inhibited with CQ or Baf. Cisplatin in itself is toxic to the cells, so a decrease in viability is expected; in this case the growth arrest is significant enough that the difference between the Cis vs. Cis + CQ/Baf groups cannot be accurately distinguished.

Figure 4G examines the same drug combinations, but this time looking at the effect on percentage of apoptotic cells, as assessed by the Annexin V/Propidium Iodide assay. Treatment with cisplatin statistically significantly increased apoptosis, and to a similar extent as in Figure 2. Further, there was no significant difference between cisplatin-treated cells and cisplatin + CQ or Baf-treated cells, indicating again that the role of autophagy is *nonprotective*. Inhibition of autophagy did not sensitize cells or cause any variations in the level of cell death. We were not yet able to confirm inhibition of autophagy with 3-methyladenine (early autophagy inhibitor), and this should be added as a future study. A western blot analysis showing p62 degradation and

LC3-I accumulation (no LC3-I to LC3-II conversion) would confirm autophagy inhibition, as would acridine orange staining showing a decrease in autophagic vesicle formation. Further, 3-methyladenine is a class III phosphatidylinositol 3-kinase (PI3K) inhibitor, so it can have off target effects and cannot be considered specific for autophagy inhibition. Therefore, one additional experiment to include in the future would be to develop a cell line with the ATG5 gene knocked out. ATG (autophagy related proteins) proteins are essential to the machinery of autophagy; ATG5 is needed specifically for phagophore formation. This genetic inhibition of autophagy would be a useful tool in assessing the role of autophagy in sensitivity to treatment with cisplatin; it is also necessary to prove that the toxic effects of a pharmacological inhibitor (such as chloroquine, bafilomycin, or 3-methyladenine) alone are not responsible for the antiproliferative effects observed here.

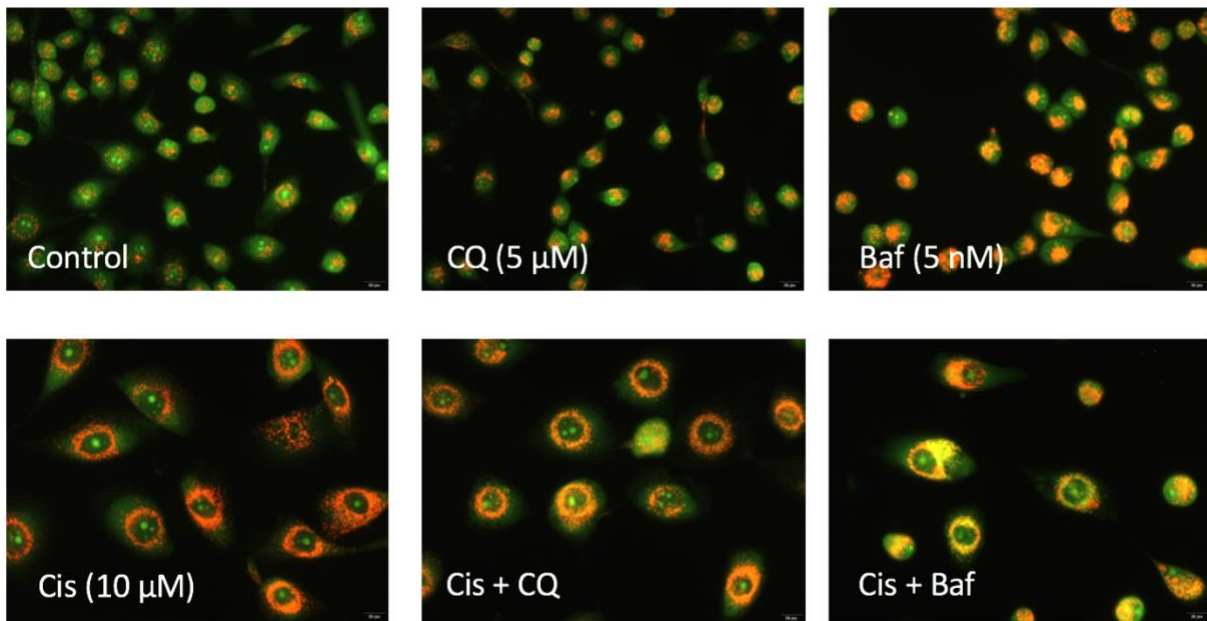


Figure 4A. Effect of chloroquine (5 μM) and bafilomycin A₁ (5 nM) on autophagy inhibition. Cells were treated with the respective drug or drug combination concurrently for 24 hours. Media was then replaced every other day. On day 3 after drug removal cells were stained with acridine orange dye (1:10,000) for 20 minutes before imaging under an inverted fluorescence microscope (n=2).

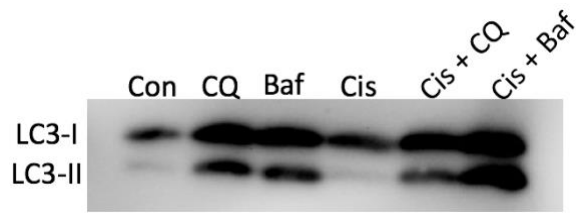


Figure 4B. Western blot analysis showing the effect of autophagy inhibition on levels of LC3-I and LC3-II. Cells were treated with the respective drug or drug combination for 24 hours before replacement with drug-free media. Collection occurred on Day 3, when Day 0 was the day of drug treatment (n=3).

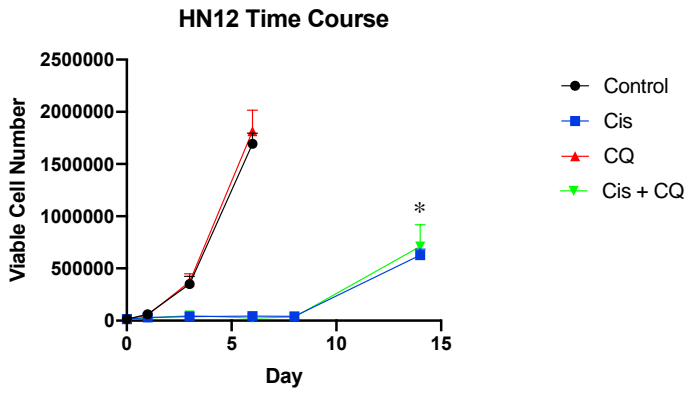


Figure 4C

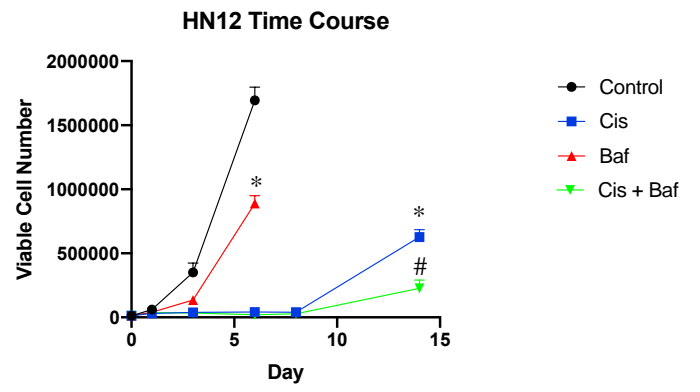


Figure 4D

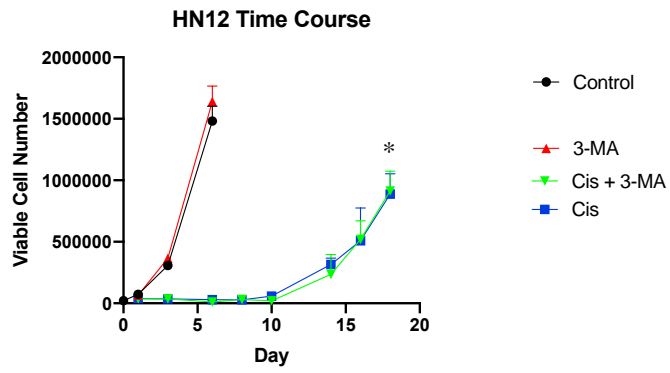


Figure 4E

Figure 4.

Cells were drug treated with respective drug or concurrent drug combination for 24 hours (5 μ M Cisplatin, 5 μ M CQ, 5nM Baf, 1 mM 3-MA). Viable cells were counted by a hemocytometer.

C) Black line indicates control group; red line indicates CQ treated group; green line indicates Cis + CQ; blue line indicates cisplatin treatment. Cells were counted on days 0 (day of drug treatment), 1, 3, 6, 8, and 14. D) Control (black); cisplatin (blue); Baf (red); Cis + Baf (green). Cells were counted on days 0 (day of drug treatment), 1, 3, 6, 8, and 14. E) Control (black); cisplatin (blue), 3-MA (red); Cis + 3-MA (green). Cells were counted on days 0 (day of drug treatment), 1, 3, 6, 8, 10, 14, 16, and 18. Counting for cells in the Control, CQ, and Baf conditions stopped on D6 due to a high level of proliferation. Data was analyzed using a two-way ANOVA; (n=3); (*) indicates significance of $p < 0.05$ as compared to controls; (#) indicates significance of $p < 0.05$ compared to cisplatin-treated group. The scales on these graphs were created in order to show a representative picture of the dataset and none of the conditions grew “0” cells; the average number of control cells between three triplicates on Day 0 is approximately 14,160.

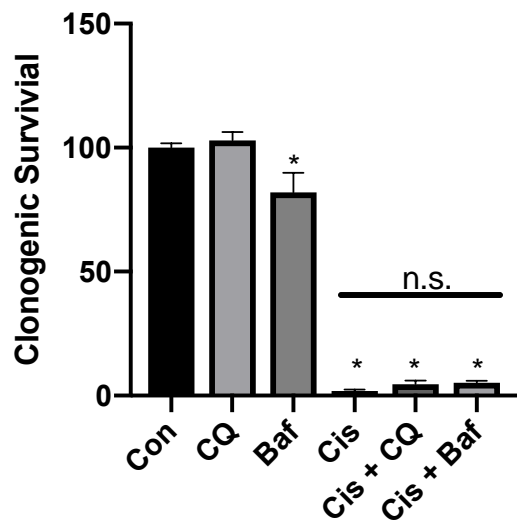


Figure 4F. Effect of inhibition of autophagy on clonogenic survival. Cells were drug treated for 24 hours (5 μ M Cisplatin, 5 μ M CQ, 5nM Baf) and allowed to grow until they formed colonies on the plate. When sufficient colonies were formed (this occurred on day 18), cells were fixed and stained and then counted. This experiment shows the percentage of colonies formed as compared to controls. Data are expressed as means of three experiments. No significant difference is found between cisplatin treated cells and cisplatin + CQ or Baf treated cells. (n=3); *p<0.05 as compared to controls.

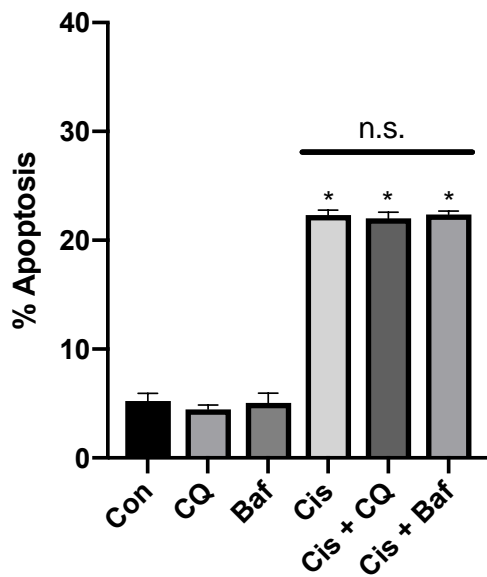


Figure 4G. Effect of inhibition of autophagy on number of apoptotic cells. Annexin

V/Propidium Iodide assay was used to assess the percentage of apoptotic cells after 24 hours of drug treatment (5 μ M Cisplatin, 5 μ M CQ, 5nM Baf). Cells were collected for flow cytometry on Day 3, where Day 0 was the day of drug treatment. Data are expressed as means of three experiments. No significant difference is found between cisplatin treated cells and cisplatin + CQ or Baf treated cells, however statistically significant increase in apoptosis was shown in these conditions as compared to controls. CQ or Baf alone did not significantly alter sensitivity. (n=3);

*p<0.05 as compared to controls.

5. Induction of senescence in HN12 cells

It is known that autophagy and senescence are interrelated pathways, and cellular enlargement and flattening were observed in the acridine orange microscopy images, so we considered the effect of senescence on HN12 cells in response to cisplatin as a cause of the growth arrest and recovery previously observed. Beta-galactosidase is a 12-sugar lysosomal enzyme that is normally active at a pH of 1-2. In senescent cells, β -galactosidase levels increase at a pH of 6. In order to visualize senescent cells, an X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) assay is used in which β -galactosidase enzyme cleaves and metabolizes X-gal, causing the cell to stain blue. After treating cells with 5 μ M cisplatin for 24 hours, cells were collected and stained on each respective day of the time course. Increased X-Gal staining was observed with cisplatin exposure, suggesting senescence induction (Figure 5A). Further, levels of senescence increased over time up to 7 days even after drug was removed. A significant increase in cell size was also observed, which is a classic morphological marker of senescence. Limitations of this assay include the fact that it is a qualitative assay and is reliant on the observer's analysis of the extent of blue staining. Therefore, it should also be assessed in a quantitative measure, as performed next.

As a quantitative measure of senescence, C₁₂FDG was used to assess levels of β -galactosidase via flow cytometry. β -galactosidase is hydrolyzed by C₁₂FDG, which emits a green fluorescence that is detectable by a flow cytometer [28]. The data from Figure 5B shows a concentration-dependent increase in β -galactosidase-positive cells in cisplatin-treated groups as compared to controls. Only groups treated with 1, 2.5, and 5 μ M cisplatin showed a statistically

significant increase. This indicates that senescence is occurring as a result of cisplatin treatment, however the maximum extent shown here is only about 20% β -galactosidase-positive, which may not be an accurate indication. Further, there seems to be an inverse relationship between increasing concentrations of cisplatin and C₁₂FDG staining; this could be due to the fact that higher concentrations are toxic to the cell and increase levels of apoptotic cells, thereby decreasing the level of senescence staining. In this experiment, cells were collected for analysis on Day 3 after drug treatment; in the future a time course for C₁₂FDG staining should be done. As indicated in the microscopy images (Figure 5A), senescence seems to peak later on in the time course, so this analysis should also be conducted on Day 5 and 7 to compare the levels of staining.

Because the C₁₂FDG did not seem to be an accurate depiction of the total level of senescence, a cell cycle analysis was performed in order to confirm that the cells were growth arrested (a key characteristic of senescent cells). Over time, cells showed an arrest in the G2/M phase of the cell cycle (Figure 5C). The sub-G1 population, which indicates the apoptotic population, was small and did not significantly change with treatment with cisplatin, which is consistent with previous data. However, this experiment was performed only once and so more repeats should be done to reach firmer conclusions for this assay. Also, growth arrest is necessary for senescence, but growth arrest in itself is not sufficient to confirm that a cell is senescent; this is just one aspect of the entire definition of senescence. DNA damaging agents (such as cisplatin) can cause cell cycle arrest distinct from senescence, so further analysis should be done to confirm the induction of significant senescence [37].

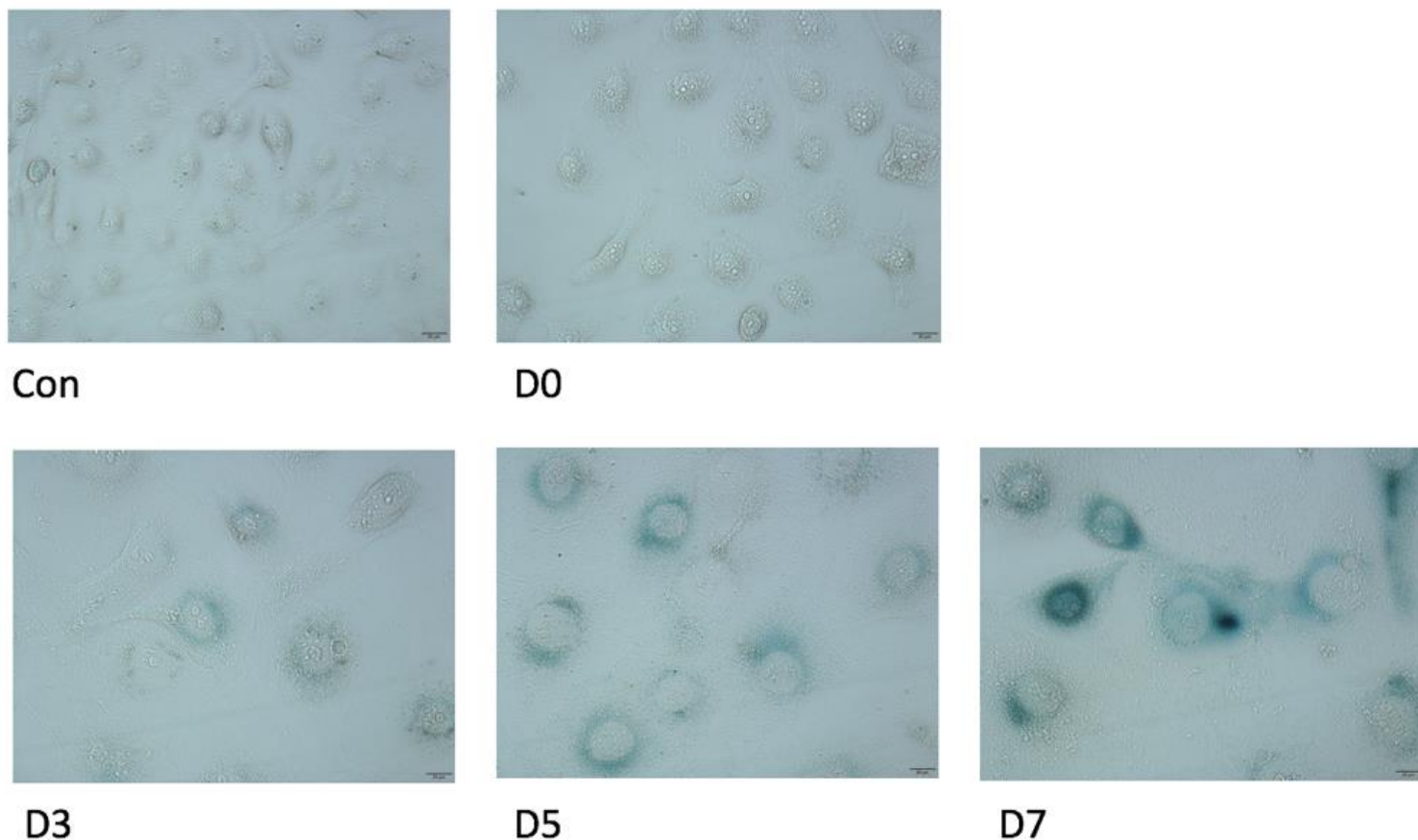


Figure 5A. Cells were treated with 5 μ M cisplatin for 24 hours before drug removal. On each respective day, cells were fixed and stained with X-Gal overnight in a CO₂ free incubator before imaging. The staining solution containing X-Gal also contains buffers that increase the lysosomal pH for B-Galactosidase detection. Day 0 indicates 24 hours after drug treatment. Blue staining indicates detection of B-galactosidase, a marker of senescence. Several images were taken of each field and most representative images are presented here. Images shows increased staining over time through Day 7 after drug removal. All images are shown on the same scale; the black bar on the bottom right corner indicates a 20 μ m scale (n=3).

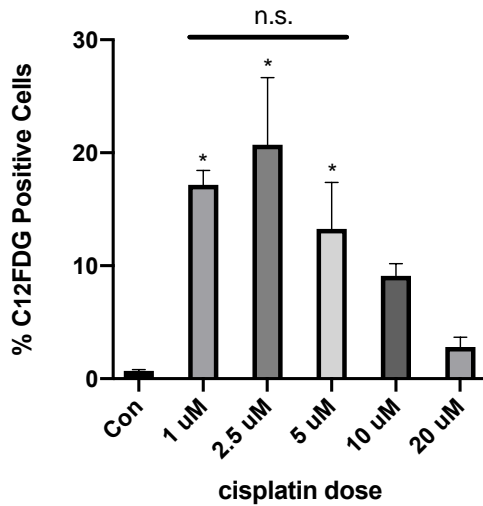


Figure 5B. Effect of concentration of cisplatin on percent C₁₂FDG-positive cells. Cells were treated with either 1, 2.5, 5, 10, or 20 μ M cisplatin for 24 hours before drug removal. Cells were collected and stained on Day 3, where D0 is the day of drug treatment. Cells were treated with Bafilomycin A₁ (1:1000) for 45 minutes to raise the lysosomal pH for B-galactosidase detection. C₁₂FDG (1:1000) was added and cells were incubated for 2 hours before collection for flow cytometry. (n=2); *p<0.05 as compared to controls. Conditions treated with 1, 2.5, and 5 μ M cisplatin were not significantly different from each other.

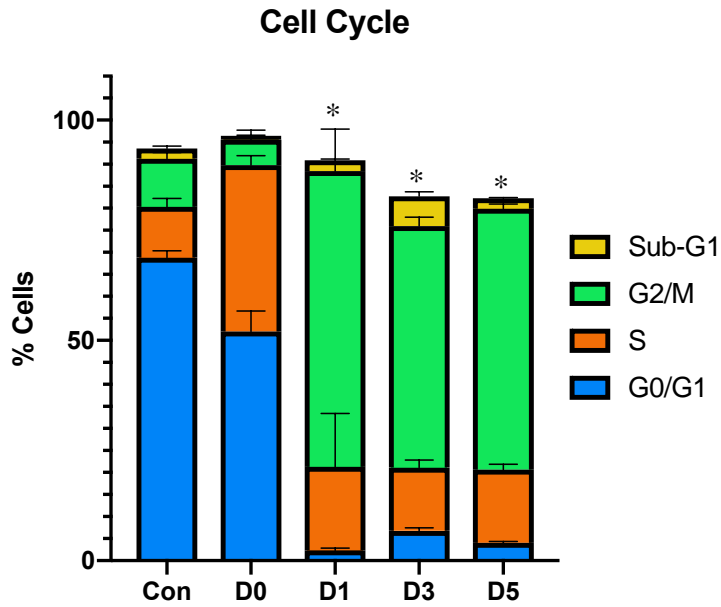


Figure 5C. Effect of cisplatin (5 μ M) treatment on cell cycle arrest. Cells were treated with 5 μ M cisplatin for 24 hours and collected on either Day 0, 1, 3, or 5, where Day 0 indicates 24 hours after drug treatment. Cells were washed and resuspended in 70% ethanol and stored until ready to analyze by flow cytometry. Blue bars indicate G0/G1 phase of the cell cycle; orange bars indicate S phase; green bars indicate G2/M phase; yellow bars indicate sub-G1 population. On days 1, 3, and 5, there is a significant increase cells arrested in G2/M phase. (n=1); *p<0.05 indicates statistically significant increase in the G2/M phase population as compared to controls. Experiment was performed once with data expressed as a mean of triplicates.

6. Effects of the senolytic ABT-263 on cell viability

Significant therapy induced senescence (TIS) was observed in response to treatment with cisplatin. As mentioned in the introduction, senescence has recently been attributed to recurrence of cancer and even metastasis. Therefore, in order to target these cells, ABT-263 (Navitoclax) was used in combination with cisplatin. ABT-263 works by inhibiting anti-apoptotic proteins such as Bcl2 and Bcl-xl, therefore leading to apoptosis of senescent cells. First, an analysis on the effect of the concentration of ABT-263 on cell viability is shown (Figure 6A). This shows 4 μM of the senolytic has a significant effect on reduction of cell viability in the HN12 cell line, and therefore 2 μM was used as an adjunct to cisplatin treatment.

Figures 6B and 6C show that treatment with Cisplatin + ABT-263 significantly decreased tumor cell viability as compared to cells treated with cisplatin alone. ABT-263 was added to the cell media on Day 5 after drug treatment; here, a marked decrease in cell viability can be seen, especially between days 5 and 7. Cells treated with both cisplatin and the senolytic showed a significant decrease in viability compared to cells treated with cisplatin alone, which extended through the entire time course. Based on the dose response shown for ABT-263, the use of 2 μM should not have had a cytotoxic effect in itself, although this experiment should be added to the time course. Further, HN12 cells (p53 null) and HN30 (p53 wild type) showed similar patterns of growth inhibition, indicating that this process may occur independent of a cell line's p53 status. In the future, this time course analysis should be extended and should also include a control population as well as cells treated with ABT-263 alone in order to confirm that ABT-263 is not having this effect alone.

In both drug-treated conditions, there seems to still be a proliferative recovery, even in the cells treated with both cisplatin and ABT-263. It is possible that autophagy may be contributing to this recovery at the later stages, or that the effects of the senolytic wear off over time and senescent cells are reappearing. It is necessary that future studies measure the amount of apoptosis occurring at separate time points after treatment with cisplatin and ABT-263, as well as amounts of autophagy and senescence. If apoptosis is occurring after treatment with ABT-263, it would suggest that the drug is working effectively. If senescence is still detectable after treatment with ABT-263 using beta-galactosidase staining, it might indicate that cells are still becoming senescent and that either ABT-263 is not acting in a specific manner, or that cells are becoming resistant to the treatment over time. If autophagy is detectable after treatment with a senolytic, it could be evidence that the role of autophagy may have switched to a protective state due to the additional stressor of the drug. These additional studies would provide a more complete picture of the effectiveness of ABT-263 and what processes may still be contributing to proliferation of both HN12 and HN30 cell lines over time. Other mechanisms of resistance could include changes in drug uptake and efflux over time, increased DNA repair, or increased detoxification of the drug; these mechanisms can be evaluated in future studies.

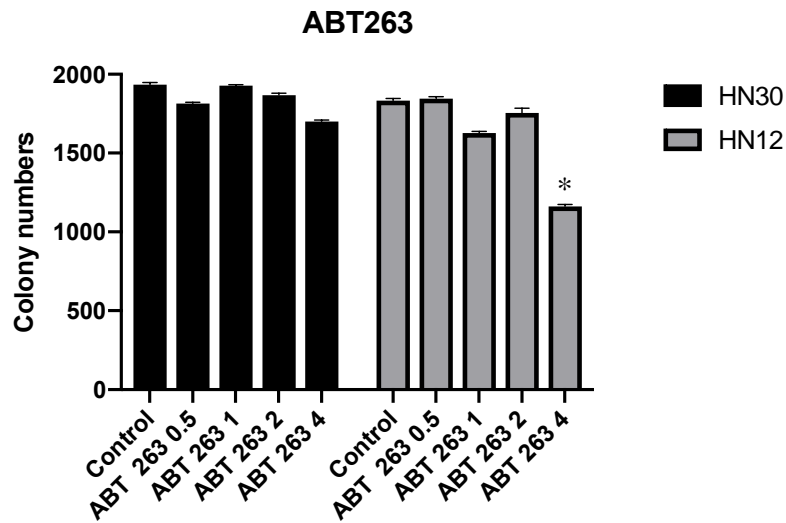


Figure 6A. The effect of ABT-263 concentration on cell viability. Each cell line was treated with either 0.5, 1, 2, or 4 μM of ABT-263. In the HN12 cell line, 4 μM concentration showed significant decrease in cell viability; therefore, 2 μM ABT-263 was used for both cell lines. (n=3); *p<0.05 as compared to controls. This experiment shows the total number of colonies formed per condition. Data generated by Fereshteh Ahmadinejad.

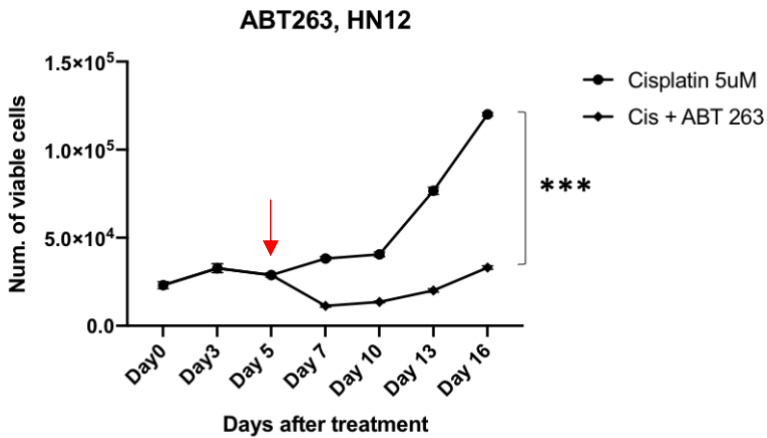


Figure 6B

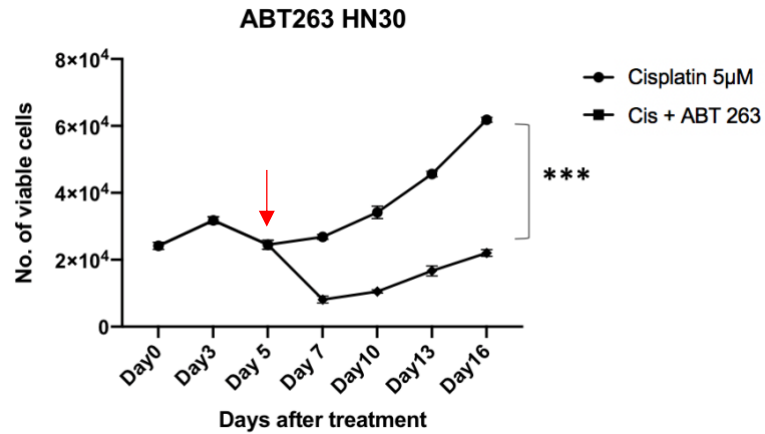


Figure 6C

Figure 6. Effect of cisplatin (5 μ M) treatment in conjunction with senolytic ABT-263 (2 μ M) on cell viability. ABT-263 was added on Day 5 after drug treatment, as indicated by red arrows. Cells were counted with a hemocytometer on each respective day through day 16 where Day 0 indicates day of drug treatment. Cisplatin treated group is indicated by circles and ABT-263 + Cisplatin group is indicated by diamonds. **B)** HN12 cells (p53 knock-down) and **C)** HN30 cells (p53 wild type). (n=3); *p<0.05 in Cis+ABT-263-treated groups as compared to cisplatin-treated groups. Data generated by Fereshteh Ahmadinejad.

Discussion

This project has examined the role of various natural cellular processes in sensitizing cancer cells to chemotherapy, in order to slow or prevent the cancer's recurrence. However, one must consider that cell communication and signaling can be much more complex than simply inhibiting any one process in order to sensitize a cell. For example, autophagy is thought to be a process activated in times of cellular stress or nutrient deprivation with the function of allowing the cell to better survive its environment. Therefore, the natural thought process would be that inhibition of this process will eliminate the cancer cell's ability to survive; we can see evidence in this project as well as in other studies, however, that this is not necessarily the case. Some cell lines and treatments elicit a nonprotective role for autophagy, where inhibiting the process has no effect on sensitizing the cells to chemotherapy whatsoever.

1. Relationship between p53 and autophagy

Another concept that is still unclear, although much work has been done, is the role of p53 in autophagy. The benefit of studying a cell line with a truncated p53 gene is that it may reflect a form of cancer in which tumor progression occurs due to a mutation in p53; this allows us to evaluate what may happen at the cellular level in patients with this mutation and what strategies for therapy may or may not be beneficial.

p53 (tumor protein 53) is considered one of the most prominent tumor suppressor genes and is one of the most commonly mutated genes in human cancers [30]. p53 is known to be a

tumor suppressor and has shown the ability to arrest cell proliferation and induce apoptosis, making chemotherapy a successful treatment for cancer [32]. This gene product has been shown to either activate or inhibit autophagy based on a variety of complex factors. The molecular process of autophagy is initiated by activation of mTOR (mammalian target of rapamycin, a serine/threonine protein kinase) protein complex, which also is responsible for regulating other processes such as protein translation and cell cycle progression [31]. mTOR complexes then phosphorylate a complex of ATG13-ULK-FIP200, which activates phagophore formation. Other various ATG proteins play a role in the nucleation (ATG12), elongation (ATG8, LC3 system), and maturation (PI3K) phases of autophagy. p53 induces autophagy by upregulating tuberous sclerosis complex (TSC2) and AMPK, which suppresses mTOR; this inhibits the ULK1 complex downstream, leading to activation of Beclin 1 (BECN1) which activates autophagy (See Figure 7).

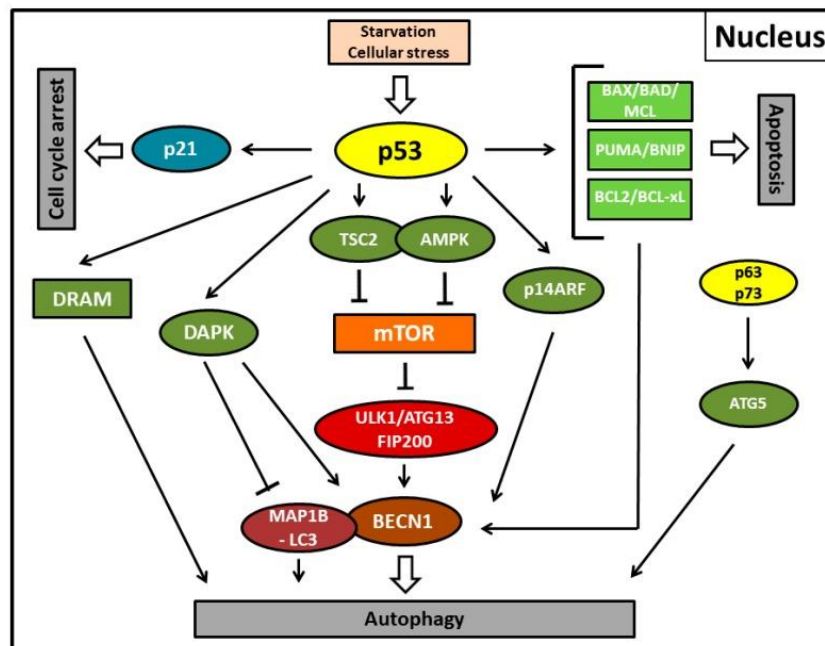


Figure 7. p53 cell signaling pathways induced by cellular stress

Mrakovcic, M., & Fröhlich, L. F. (2018). p53-Mediated Molecular Control of Autophagy in Tumor Cells. *Biomolecules*, 8(2), 14.

Previous research has shown that cisplatin (a form of cellular stress) upregulates autophagy as a cytoprotective mechanism. Further, p53 has been shown to induce this autophagic response; however, in the HN12 cell line (p53-null), autophagy is still significantly induced in response to 24 hours of cisplatin treatment. This indicates that the role of p53 is very complex. According to Galuzzi et. al., resistance to cisplatin commonly involves inactivation of p53; they found that in those with ovarian cancer, patients with wild type p53 benefitted more from chemotherapy than those with p53 mutations, and that loss of p53 in this case was related to tolerance to damage caused by cisplatin, increasing resistance to treatment [29].

According to studies done by Tasdemir et. al., p53 can elicit either pro-autophagic or anti-autophagic responses; it was found that p53 translocated in the nucleus caused autophagy through transcriptional effects, and p53 in cytosol inhibited autophagy, although both utilized the same signaling pathway [33]. Further, p53 activation has been shown to *induce* autophagy in response to stress (transcription independent & dependently), which may lead to cell death, or increase cell survival; also, basal levels of p53 have been shown to *inhibit* autophagy in the absence of stress signals [30]. This points to the importance of distinguishing between *basal levels* of p53 and *activated* p53, where basal p53 usually promotes autophagy and high levels usually promote cell death [30].

Tasdemir et. al also showed that p53 *loss* increased autophagy associated with AMPK activation and mTOR suppression, and this was inhibited by the knockdown of autophagy genes like ATG5, ATG10, and beclin 1 [30]. They also found that expression of autophagy-related transcripts of wild-type and p53-deficient cells were comparable. Additionally, the role of p53 and autophagy had to do with the nature of the stress signal; for example, genotoxic stress versus ER stress or starvation could potentially cause p53 to activate or inactive autophagy.

Another interesting study from Mrakovcic et. al. showed that when p53 wild type and p53-deficient mouse embryonic fibroblasts were treated with Doxorubicin, autophagy was induced, and that other p53 family members such as p73 and p63 may have compensated for the loss of p53 [31]. Perhaps a better conclusion for the role of p53 in autophagy is that it is not an “on or off” switch for autophagy, and as stated by Tasdemir et al, “Perhaps p53 operates within a broader regulatory network to define autophagic control.”

All of these studies point to the fact that the role of p53 in autophagy is not yet clear and that multiple factors contribute to the form and activation of autophagy, including the type and strength of cellular stressor, the environment of the cell, and the length of time under stress. Although there is much variability and complexity in the mechanisms of responses to chemotherapy, more work can be done to better define the various roles of these proteins and genes in order to formulate better treatments in the clinic.

2. Relationship between p53 and senescence

p53 also plays a role in senescence in that it induces tumor growth suppression and induces senescence. It has been shown that the loss of p53 in senescent cells may allow them to recover from senescence. Further, loss of p53 enhances the senescence associated secretory phenotype (SASP) and pro-tumorigenic proliferation [23]. This may be one potential action in the HN12 cell line which allow them to eventually recover from senescence and proliferate, even after treatment with a senolytic.

Additionally, this project has provided some evidence that sensitization to cisplatin after treatment with the senolytic ABT-263 occurs independent of p53 status; however, the HN12 and

HN30 cells lines shown in this project are not isogenic and there are therefore limitations when comparing the two. Further evidence of this pattern is shown by data produced from the laboratory of Dr. Hisashi Harada (Figure 9). The data from Figure 9 shows two isogenic cell lines, HN30 wild type compared to HN30 with a p53 knockdown (shp53). The data shows that cisplatin induces senescence in both the HN30 wild type and shp53 cell lines, as indicated by β -galactosidase staining (Figure 2B). Further, both cell lines are sensitive to treatment with ABT-263, as shown by reduced cell viability in a clonogenic assay (Figure 2C). This data is valuable because these two cell lines are isogenic, however it is possible that there is residual p53 due to the gene not being completely eliminated. This work is consistent with the findings of this project and may be an indicator of the potential clinical relevance of ABT-263 in head and neck cancers.

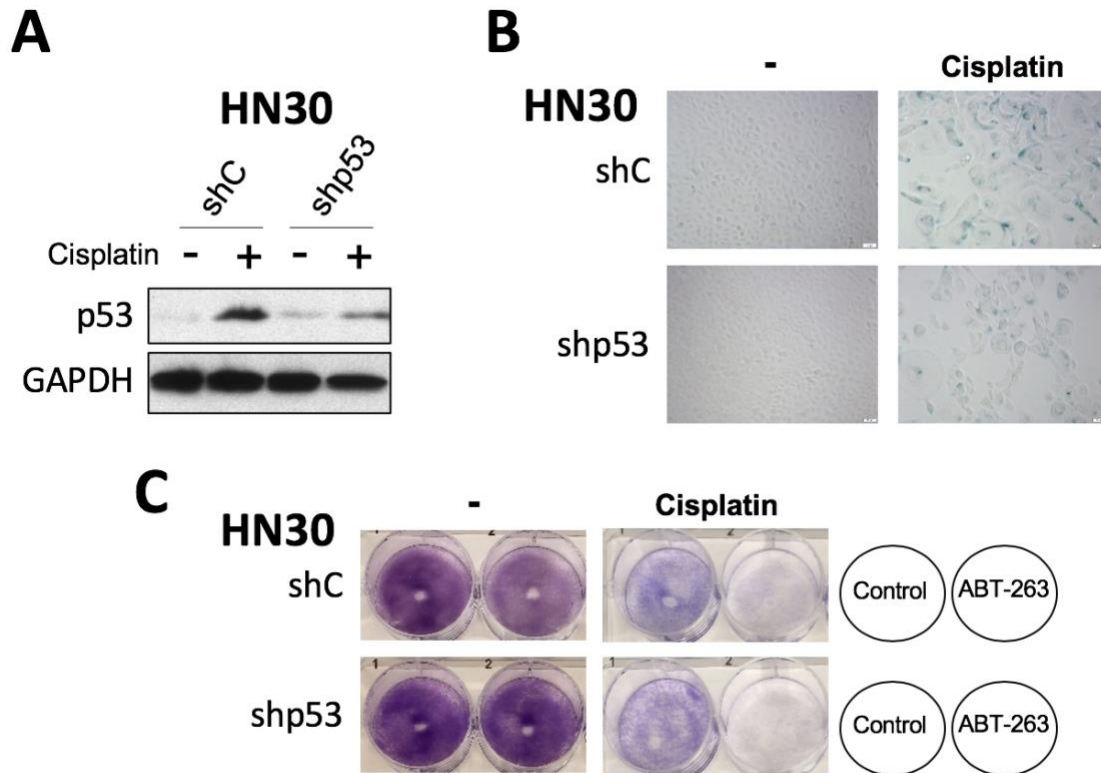


Figure 8. Down-regulation of p53 in HN30 cells does not affect cisplatin-induced senescence induction and ABT-263 sensitivity. (A and B) HN30 shp53 and shC (scrambled control) cells were treated with cisplatin (5 μ M) for 5 days. The levels of p53 were determined by Western blots (A), and senescent cells were detected by β -gal staining (B). (C) Following cisplatin treatment, cells were treated with ABT-263 (1 μ M) for 24 hrs, and cell viability was determined by crystal violet staining. Data was generated by the laboratory of Dr. Hisashi Harada, Virginia Commonwealth University.

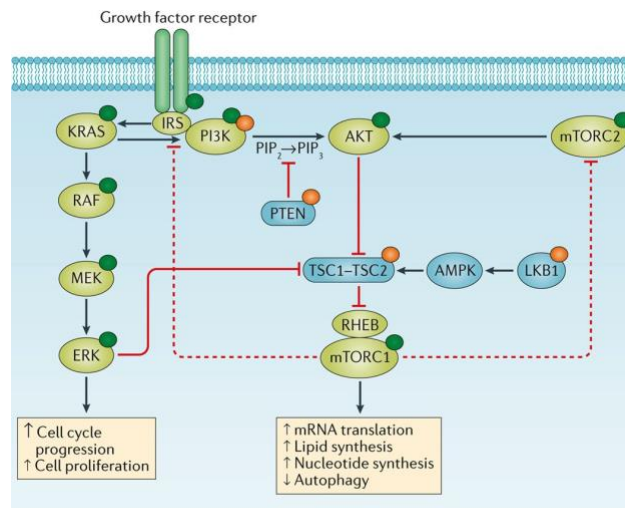
3. Autophagy and Senescence

Another interesting aspect of autophagy and senescence is their interrelationship, which is still not completely clear. Both processes are similar in that they can be activated in times of external stressors such as chemotherapy and radiation in order to protect the cell and evade cell death. Studies have previously stated that autophagy may be required for senescence to occur and that mTOR promotes senescence via autophagy inhibition [32]. A study by Young et. al suggested that oncogene-induced senescence in fibroblasts was dependent on autophagy because inhibition of autophagy delayed senescence [38]. This is consistent with the evidence in this project; cells showing increased acridine orange staining due to induction of autophagy also had a distinct flattened, enlarged morphology indicative of senescence, which may be evidence that autophagy and senescence are occurring in the same cell. However, more evidence has shown that although interference with autophagy delays senescence, it is not essential for senescence to occur; further, even if autophagy causes senescence to occur, senescence can be autophagy-independent well after its initiation. Studies from our lab have shown that stress-induced DNA damage induces senescence and that this does not require autophagy [36].

Based on this, it is likely that autophagy and senescence are linked, but are not necessarily dependent on each other. In this project, significant autophagy and senescence appear to be occurring simultaneously; based on the evidence discussed, it is possible that the inhibition of autophagy via pharmacological inhibitors also has an effect in delaying senescence. More work should be done to analyze this interrelationship; for example, evaluating levels of senescence over time after treatment with chloroquine. This understanding could be beneficial in shaping our approach to sensitizing cancer cells to treatment.

4. Limitations of Autophagy Inhibitors

One limitation that has been mentioned in the studies of autophagy inhibition, is the nonspecific effects of certain drugs such as 3-methyladenine. In addition to its role as a pharmacological inhibitor of autophagy, 3-methyladenine can have alternate downstream effects in the cell due to its action as a class III phosphatidylinositol 3-kinase (PI3K) inhibitor. PI3K inhibition blocks the production of PI3P which recruits other ATG proteins in order to initiate autophagy [35].



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Figure 9. PI3K Cell Signaling Pathway

Janku, F., Yap, T. & Meric-Bernstam, F. Targeting the PI3K pathway in cancer: are we making headway?. *Nat Review Clinical Oncology* 15, 273–291.

Therefore, PI3K inhibition plays an important role in blocking autophagosome formation. However, studies have also shown that 3-MA induces caspase-dependent cell death and that this is independent of autophagy inhibition [34]. In addition, studies from Wu et. al. have shown that long-term treatment with 3-MA actually promoted autophagy flux in nutrient-rich conditions, but

that in conditions of cell starvation, 3-MA still showed autophagy suppression [35]. These effects may have to do with 3-MA's effects on both Class I and Class III PI3K. Class I PI3K phosphorylates PI to generate PI3P. When PI3K is activated, it activates AKT via kinases mTOR (complex 2) and PDK1, which then activates the tuberous sclerosis complex and Rheb, which activates mTOR (complex 1) and leads to suppression of autophagy (See Figure 8) [35].

These studies suggest that it is critical to be cognizant of the fact that drugs like 3-MA may have mechanisms of action that cause downstream effects other than the desired mode of action (autophagy inhibition). It also points to the importance of using alternate methods of autophagy inhibition, such as an autophagy-gene knockdown.

5. Strategies for Sensitization and Conclusions

Based on the data collected in this project, autophagy inhibition as a strategy for sensitization to cisplatin in head and neck cancer cells would be ineffective. However, treatment with a senolytic seems to have potential therapeutic benefits due to the significant decrease in HN12 and HN30 cell viability after treatment with cisplatin and ABT-263. Additional studies should be conducted to assess the mechanisms that may be contributing to the eventual recovery and proliferation of the cells after treatment with the senolytic. For example, if cells are escaping treatment and still becoming senescent after several days of senolytic treatment, it may be beneficial to try sequential treatments over time when cells are expected to recover. Based on the time course in Figure 6A, for instance, drug treatments on day 0 and then on day 10 may prevent proliferation; however, this may have a limitation in increasing toxicity in the body.

Additionally, levels of autophagy should be measured in the days following treatment with ABT-263; if autophagy is increasing then it may suggest that autophagy is switching to playing a more protective role in response to this anti-apoptotic protein inhibitor, in order to evade apoptotic cell death. In this case, a treatment strategy of ABT-263 and chloroquine might show some benefit, either in combination or sequentially. Based on the results shown in this project, chloroquine seems to be the best pharmacological inhibitor autophagy for use in this cell line due to potential off target effects of bafilomycin and 3-methyladenine; chloroquine seems to be the drug most specific to the process of autophagy. After these mechanisms are more firmly more established, they may be potentially applied to the treatment of tumor-bearing animals to assess whether the treatments translate *in vivo*, and also to assess whether the animal's immune system plays a role in this process. More work needs to be done to further elucidate the mechanisms contributing to eventual survival and proliferation of cells after treatment with cisplatin, but the use of the senolytic ABT-263 is a promising start.

In conclusion, although much literature points to the cytoprotective role in cancer chemotherapy, autophagy can also have a nonprotective role, as shown by the evidence that pharmacological inhibition of autophagy does not sensitize HN12 cells to cisplatin. Therefore, autophagy is not necessarily an intrinsic mechanism of resistance. Further, chemotherapy promotes primarily autophagy and senescence rather than apoptosis, and treatment with the senolytic ABT-263 sensitizes cancer cells to treatment regardless of p53 status. This may be a potential therapeutic mechanism, however more work needs to be done to better sensitize HN12 cells to cisplatin and prevent cell proliferation.

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Contributions

Data from the HN30 cell line was generated by Fereshteh Ahmadinejad from the laboratory of Dr. David Gewirtz at Virginia Commonwealth University Massey Cancer Center. shp53 data from the HN30 cell line was contributed by the laboratory of Dr. Hisashi Harada, also from Virginia Commonwealth University Massey Cancer Center. I thank both Dr. Harada and Fereshteh for their valuable contributions to this project. Services in support of this research project were provided by the VCU Massey Cancer Center Flow Cytometry Core, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30 CA016059.

Vitae

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