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Combining Noxa-Inducing Drugs with ABT-263 to Efficiently Increase Cell Death in Head and Neck Squamous Cell Carcinoma (HNSCC)

Sung Woo Kim

COMBINING NOXA-INDUCING DRUGS WITH ABT-263 TO EFFICIENTLY INCREASE CELL DEATH IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

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

By

SUNG WOO KIM
Virginia Tech, B.S. Biological Sciences, 2013

Director: HISASHI HARADA, PH.D.
SCHOOL OF DENTISTRY
PHILIPS INSTITUTE FOR ORAL HEALTH RESEARCH

Virginia Commonwealth University
Richmond, Virginia
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Abbreviations

HNSCC: Head and neck squamous cell carcinoma

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

BCL-2: B-cell lymphoma 2

MCL-1: Myeloid cell leukemia 1

BCL-X_L: B-cell lymphoma-extra large

BAK: BCL-2 homologous antagonist killer

BAX: BCL-2 associated X protein

BAD: BCL-2-associated death promoter

BID: BH3 interacting-domain

CDK2: Cyclin-dependent kinase 2

HPV: Human papillomavirus

shRNA: Short hairpin RNA

cDNA: Complementary DNA

E. coli.: Escherichia coli

TNF: Tumor necrosis factor

TNFR1: Tumor necrosis factor receptor 1

FasL: Fas ligand

FADD: Fas-associated death domain

MOMP: Mitochondrial outer membrane permeabilization

APAF1: Apoptotic protease activating factor 1

BH: BCL-2 homology

DMEM: Dulbecco’s modified eagle medium

FBS: Fetal bovine serum

PI: Propidium iodide

PARP: Poly (ADP-ribose) polymerase

ABSTRACT

COMBINING NOXA-INDUCING DRUGS WITH ABT-263 TO EFFICIENTLY INCREASE CELL DEATH IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

By: Sung Woo Kim, B.S.

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

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Major Director: Dr. Hisashi Harada, Ph. D
VCU School of Dentistry
Philips Institute for Oral Health Research

Head and neck cancer is the sixth leading cancer worldwide. Head and neck squamous cell carcinoma (HNSCC) accounts for more than 90% of incident cases. Despite intense, multimodality treatment regimens for HNSCC including surgery, chemotherapy, and radiation, little progress has been made over the past 30 years in improving overall survival rates. Tumor cell death induced by both conventional and targeted chemotherapy is often mediated by the BCL-2 family-dependent mitochondrial apoptotic pathway. However, initiators of this apoptotic pathway, such as p53, are more than 50% of the time mutated or deleted in HNSCC rendering the disease refractory to treatment. To counter such resistance, direct therapeutic targeting of the BCL-2 family is

conceptually appealing. For this purpose, we use three clinically-available drugs: cisplatin, fenretinide, and ABT-263 (navitoclax). Both cisplatin and fenretinide are known to induce a BH3-only pro-apoptotic protein, Noxa, which binds to and inactivates multi-domain anti-apoptotic protein MCL-1 and release from its interaction with multi-domain pro-apoptotic protein BAK, followed by the phosphorylation via CDK2 for the proteasome-mediated degradation. Activated BAK can now go through conformational change for the oligomerization at the outer membrane of the mitochondria to release cytochrome c into the cytosol and induce caspase-dependent apoptotic cell death. ABT-263 directly binds to multi-domain anti-apoptotic proteins, such as BCL-2 and BCL-X_L, to inhibit their activity and leads to the activation of multi-domain pro-apoptotic protein BAX to induce apoptosis.

We hypothesize that combining the Noxa-inducing drugs (cisplatin or fenretinide) along with ABT-263 can efficiently induce BAX and BAK activation and significantly increase cell death in HNSCC cells by simultaneously inhibiting the activity of MCL-1, BCL-2, and BCL-X_L. Combination-induced treatments in four cell lines (HN8, HN30, HN31, and UMSCC1) tested led to significant increase in apoptotic cell death. Cisplatin and ABT-263 combined treatment is inducing the expression of Noxa and leading to increase in apoptosis in HN30, HN31, UMSCC1, but not HN8. Similarly, fenretinide and ABT-263 combined treatment is inducing the expression of Noxa in all four cell lines tested and is largely relying on expression of Noxa.

INTRODUCTION

1.1 Cancer

Cancer is a genetic disease that is caused by an uncontrolled division of abnormal cells in a part of the body due to altering genes. Cancer has a major impact on society in the United States and across the world. The statistics have estimated 1,685,210 new cases of cancer to be diagnosed in 2016 in the United States and 595,690 will die from this leading cause of death [www.cancer.gov]. Among various types of cancers, the deadliest types include stomach, lung, liver, colorectal, breast, and esophageal cancer [www.who.org]. With this increasing number of deaths and new cases each year, it is extremely critical that we understand and make significant progression in molecular mechanisms of tumorigenesis and cell death.

While cancer can arise from genetic alterations, there are other factors that people cannot control, such as age and family history. Other environmental risk factors also include, but are not limited to tobacco, radiation, sunlight, and alcohol. Use of tobacco products is the leading cause of cancer and of death from cancer because tobacco products and secondhand smoke contain many chemicals that damage DNA. Tobacco use causes many types of cancer, including cancer of the lung, larynx (voice box), mouth, esophagus, throat, bladder, kidney, liver, stomach, pancreas, colon and rectum, and cervix, as well as acute myeloid leukemia. People who use smokeless tobacco (snuff or chewing tobacco) have increased risks of cancers of the mouth, esophagus, and pancreas [www.cancer.gov]. Radiation of certain wavelengths are also called ionizing radiation, which has enough energy to damage DNA and cause cancer. High-energy radiation, such as x-rays, gamma

rays, alpha particles, beta particles, and neutrons can also damage DNA and cause cancer. While these are some of the primary causes of cancer, there are many other risk factors of developing cancer, such as obesity, human immunodeficiency virus (HIV), human papillomavirus (HPV), hormones, infectious agents, and age [www.cancer.gov].

1.2 Head and neck cancer

Head and neck cancer is a general term used to describe a number of different deadly tumors that arise from squamous cell lining the epithelium of the oral cavity, nasal cavity, pharynx, larynx, paranasal sinuses, and salivary glands, hence called head and neck squamous cell carcinoma (HNSCC) [1]. HNSCC is the sixth leading cancer worldwide. Head and neck cancers account for approximately three percent of all cancers in the United States, and these cancers are nearly twice as common among men as they are among women [2,3]. Symptoms of head and neck cancers include a lump or a sore that does not heal nor go away, difficulty in swallowing, and a change or hoarseness in the voice [www.cancer.gov]. Individuals who are older than 50 are most vulnerable to HNSCC and many of them have been exposed to one or more of the risk factors [www.cancer.gov]. The survival rate for individuals diagnosed with head and neck cancer has not changed significantly over the past 50 years and have been remaining at 50 percent [www.mcancer.org].

Alcohol and tobacco are the two most important risk factors for head and neck cancers, which accounts for at least 75 percent of all head and neck cancers [4]. These two highest risk factors mainly affect the oral cavity, oropharynx, hypopharynx, and larynx [5].

Alcohol and tobacco are not risk factors for salivary gland cancers. Another high-risk factor for some types of head and neck cancers, particularly oropharyngeal cancers that involve the tonsils or base of the tongue is human papillomavirus (HPV), especially HPV-16 [6-8]. In the United States, the incidence of oropharyngeal cancers caused by HPV infection is increasing, while the incidence of oropharyngeal cancers related to other causes is falling [6].

Treatment of the head and neck cancers may vary depending on its location and the stage of cancer. Cancer staging is the process of finding out how much cancer is in a person's body and where it is located. For most types of cancer, doctors use staging information to help plan treatment and to predict a patient's prognosis [www.cancer.org]. Although each person's situation is different, cancers with the same stage tend to have similar outlooks and are often treated with the same way. In order to stage cancers, doctors use TNM classification system as a tool to stage different types of cancer based on certain standards, and place the tumor from stage zero to four. The TNM classification system considers the size of tumor, possible spread to lymph nodes, and possible metastasis to other parts of the body [www.cancer.org]. At stage 0, abnormal cells are present but have not spread to nearby tissue. Also, called carcinoma in situ, or CIS. CIS is not cancer, but it may become cancer. Stage I tumors are estimated to be 2 cm in diameter or smaller, and no other cancer cells are present in surrounding tissues or distant structures. Stage II tumors measure 2-4 cm in diameter, yet no cancer cells are present in surrounding tissues or distant structures. Stage III tumors have grown even larger to 4 cm in diameter or greater. Stage IV is further divided into three subtypes: A, B, and C. Stage IV-A means that the tumor is in any size, but is not found in lymph nodes or other parts of the body. Stage IV-B tumors

invade deeper tissues and have spread to lymph nodes, but not into distant areas. Stage IV-C tumors have spread to lymph nodes as well as other distant parts of the body [www.cancercenter.com].

As already mentioned, treatment options may vary depending on which stage the tumor is in. For tumors that are in stage I-II, either radiation or surgery is needed. For further progressed patients at stage III-IV, treatments may include surgery, chemotherapy, and radiotherapy. Some of chemotherapeutic drugs that are used to treat head and neck cancers include docetaxel, gemcitabine, fluorouracil, carboplatin, and cisplatin.

1.3 Cell Death

Three major types of cell death include apoptosis, necrosis, and autophagy. Apoptosis is a type I programmed cell death that occurs in multicellular organisms as a result of caspase activation. This leads to the morphological changes of cells and is characterized by cell shrinkage, plasma membrane blebbing, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis in general, is a highly regulated and controlled process which confers advantage during an organism's life cycle. For example, separation of humans' fingers and toes is regulated by apoptosis during the embryological development. Autophagy is another type of cell death that is caspase-independent. It is an intracellular degradation system that delivers the cytoplasmic constituents to the lysosome. The most typical trigger of autophagy is the nutrient starvation. Necrosis results in the premature death of cells in living tissue. Necrosis is

caused by external factors to the cell, such as infections, toxins, or trauma. Unlike apoptosis, necrosis is almost always detrimental to an organism and can be even fatal.

In order for human body to maintain proper homeostasis, apoptosis is extremely important and must be in absolute control to balance out the amount of cell proliferation to avoid cancer development. Excessive apoptosis causes atrophy, whereas an insufficient amount of apoptosis results in uncontrolled cell proliferation, such as cancer. Some factors like Fas receptors and caspases promote apoptosis while some members of the BCL-2 family of proteins inhibit apoptosis.

Apoptosis can be divided into two main pathways: intrinsic and extrinsic pathways [15]. The intrinsic pathway is characterized by permeabilization of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex that is known as apoptosome and initiates activation of the caspase cascade through caspase-9. This pathway is often triggered by harmful stimuli, such as chemotherapeutic drugs. When cytochrome c is released from the mitochondria, it binds to the apoptotic protease-activating-factor 1 (APAF-1) to form apoptosome. This complex then binds and forms another complex with pro-caspase 9 and cleaves it to release its active form. Fully activated caspase-9 then leads to the activation of caspase-3. The extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors and these ligands include tumor necrosis factor-α (TNF- α) and Fas ligand (CD95), which leads to the formation of the death-inducing signaling complex (DISC). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors results in activation of

the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 [16].

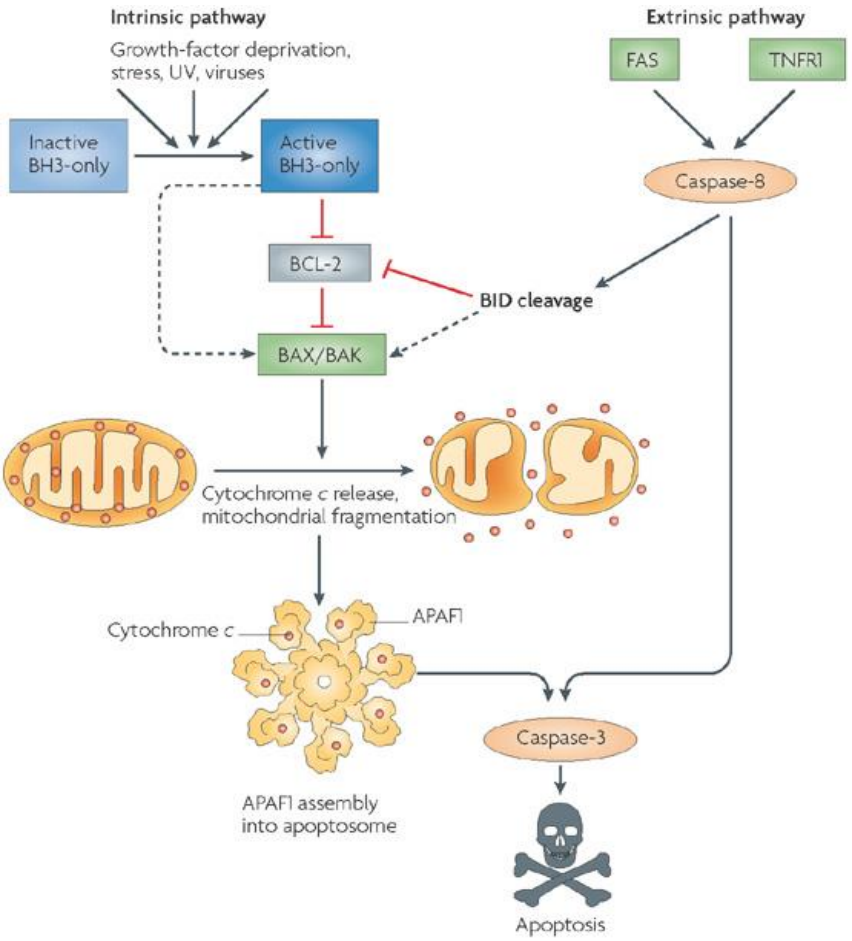


Figure 1: Simplified diagram of the intrinsic and extrinsic pathways of apoptosis [16].

1.4 BCL-2 family

The BCL-2 family proteins consist of a number of proteins that share the BCL-2 homology (BH) domains, and they play a significant role in the regulation of apoptosis, a form of programmed cell death, through mitochondrial pathway. Proteins in this family either promote or inhibit apoptosis by governing mitochondrial outer membrane permeabilization (MOMP). The family is subdivided into three classifications based on its functions and by which BH domain it consists. As of today, four groups of BH domain have been identified and proteins belonging to this family contain either BH1, BH2, BH3, or BH4 domain. The BCL-2 family consists of three different groups of proteins: multi-domain pro-apoptotic, multi-domain anti-apoptotic, and finally, BH3-only pro-apoptotic proteins. Multi-domain pro-apoptotic includes BAX and BAK, multi-domain anti-apoptotic includes BCL-2, MCL-1, BCL-$X_{L,}$ BCL-W, and A1. BH3-only pro-apoptotic proteins include BAD, BIM, BID, and Noxa.

Upon activation of BAX and BAK, which contain all four BH domains, they eventually go through the conformational change and oligomerize, then cytochrome c is released into the cytosol from the mitochondria to trigger apoptosis through caspase-dependent event, so called MOMP. Multi-domain anti-apoptotic proteins act to inhibit the function of pro-apoptotic proteins, such as BAX and BAK by the protein-protein interaction and prevent MOMP or release of cytochrome c into the cytosol. BH3-only pro-apoptotic proteins share homology only with the BH3 domain and they tend to inactivate the activity of the anti-apoptotic proteins, such as BCL-2, BCL-X_L and MCL-1. To inactivate the activity of these anti-apoptotic proteins, the BH3-only protein's BH3

amphipathic helix binds to the hydrophobic pocket of anti-apoptotic proteins (BCL-2, BCL-XL, and MCL-1), which prevents these anti-apoptotic proteins from interacting with BAX and BAK for the pore formation to release cytochrome c. Some of the proteins within this group, such as BID and BIM, have a unique ability to directly activate BAX and BAK to induce caspase-dependent apoptosis. Due to differences of BH3-only protein's BH3 domains and the groove in which they bind to, some BH3-only proteins, such as Noxa is very selective and has high affinity to MCL-1 (myeloid cell leukemia 1) over any other anti-apoptotic proteins, such as BCL-X_L and BCL-2.

Without an apoptotic stimulus or cells in a healthy state, BAX is located in the cytosol and remain inhibited by variety of anti-apoptotic proteins. However, upon activation through apoptotic stimulus, such as DNA damage, it gets conformational change to expose mitochondrial targeting sequence, and is sequestered to the outer membrane of the mitochondria. On the other hand, BAK is normally located at the outer membrane of the mitochondria and remain inhibited by anti-apoptotic proteins, such as MCL-1 and BCL-X_L. The BH grooves exist in all anti-apoptotic BCL-2 proteins. This groove of anti-apoptotic proteins prevents the formation of pore at the outer membrane of the mitochondria by binding to the BH3 domain of BAX or BAK and interferes with their conformational change for activation. There are differences in BH3-only protein's BH3 domains and grooves in which they bind to. For example, BIM and BID are capable of binding to all anti-apoptotic proteins, whereas Noxa is extremely selective and has much higher affinity to MCL-1 while has lower affinity to other anti-apoptotic proteins.

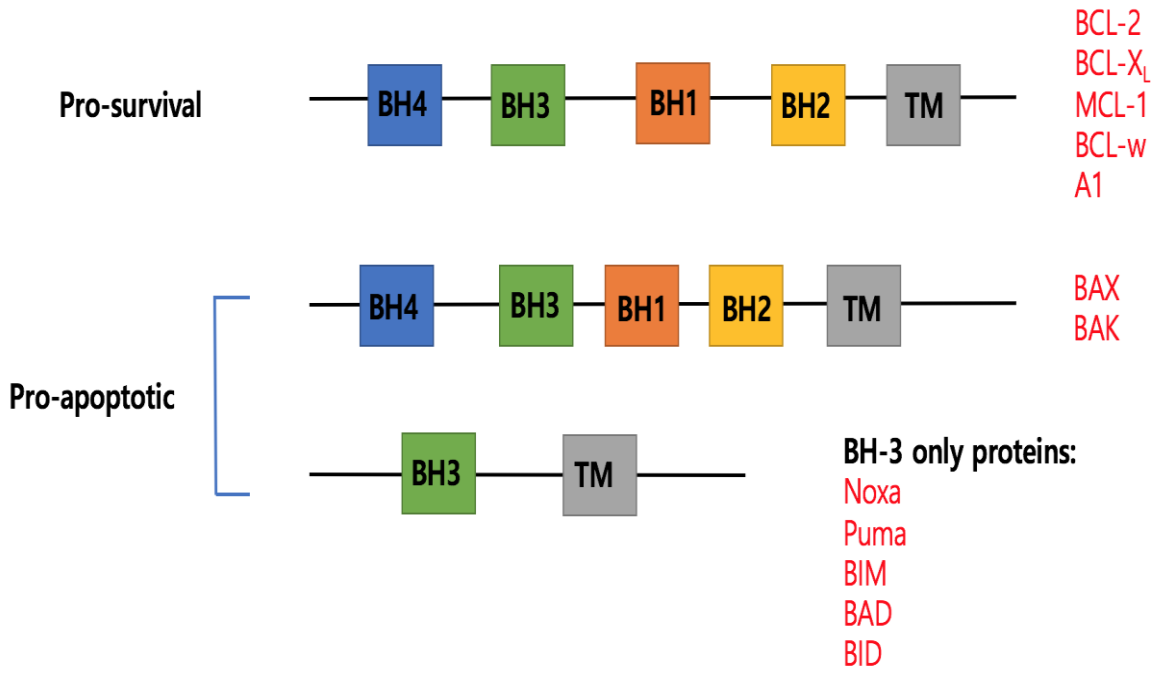


Figure 2: The list of BCL-2 family proteins. All BCL-2 family proteins contain BH3 domain.

1.5 MCL-1 and Noxa

MCL-1 (myeloid cell leukemia 1) was first discovered in 1993 in the differentiating myeloid leukemia cells [17]. This protein encoded by this gene belongs to the BCL-2 family proteins. Its major role is regulation of apoptosis and cell survival in both normal and tumor cells. This protein is composed of 350 amino acids in human and lacks in BH4 domain, while BCL-2 and BCL-X_L contain BH1, BH2, BH3, and BH4 domain. The predominant

function of MCL-1 is to prevent apoptosis through its protein-protein interaction with multi-domain pro-apoptotic proteins, such as BAK. In the C-terminal portion of MCL-1, there is a transmembrane domain that functions to localize MCL-1 to the outer membrane of mitochondria [18]. The N-terminus of MCL-1 is unique amongst the BCL-2 family, in that it is rich in putative regulatory residues and motifs. These include the sites for ubiquitination, cleavage, and phosphorylation, which influence the protein stability, localization, dimerization, and function [19]. The PEST domains at the N-terminus of MCL-1 contain phosphorylation sites for protein kinases, such as JNK (c-JUN N-terminal kinase), ERK (extracellular signal-regulated kinase), GSK3 (glycogen synthase kinase 3), CDK1 (cyclin-dependent kinase 1), and CDK2 (cyclin-dependent kinase 2). For instance, when the GSK3 pathway is activated by the decrease in activation of AKT, it leads to phosphorylation of MCL-1 and ultimately degradation. In contrast to GSK3 pathway, ERK-mediated pathway phosphorylation of MCL-1 stabilizes the MCL-1 protein by phosphorylating at Thr 163 in the PEST region, stimulated by 12-O-tetradecanoylphorbol acetic acid (TPA)-induced activation of extracellular signal-regulated kinase (ERK) [35]. When healthy cells undergo apoptotic events due to cytotoxic stimulus, MCL-1 is often down-regulated as a result of caspase cleavage. These activated caspases have the ability to cleave MCL-1 at the N-terminus that is within the PEST domain, and lead to its loss of function as an anti-apoptotic protein to interact with BAK to prevent apoptosis. Overall, MCL-1 can be either phosphorylated for the proteasome-mediated degradation or by cleavage by caspase to affect its function.

Noxa is one of the BH3-only pro-apoptotic proteins that is identified as a target gene of p53 (REF). It was first identified in 2000 and is composed of 54 amino acids in human. Under the cytotoxic stimulus, such as DNA damage, Noxa is up-regulated, binds to one of the anti-apoptotic BCL-2 family proteins MCL-1 at the BH3 domain and recruits at the membrane of the mitochondria from cytosol. Upon interaction between Noxa and MCL-1, phosphorylation of MCL-1 occur at Ser64/Thr70 sites and get ubiquitinated for subsequent proteasome-mediated degradation. This phosphorylation event is primarily regulated by CDK2 (cyclin-dependent kinase 2) [20]. This up-regulation of Noxa can be either p53-dependent and also p53-independent. For example, application of cisplatin to cause DNA damage triggers p53 to induce Noxa expression, whereas mitogenic stimulation to induce Noxa through PKC (protein kinase c) is independent of p53. Upon up-regulation of Noxa and subsequent activation of BAK, BAK can now go through conformational change and oligomerize to form a pore in the outer membrane of the mitochondria, releasing cytochrome c into the cytosol for further caspase-9 activation.

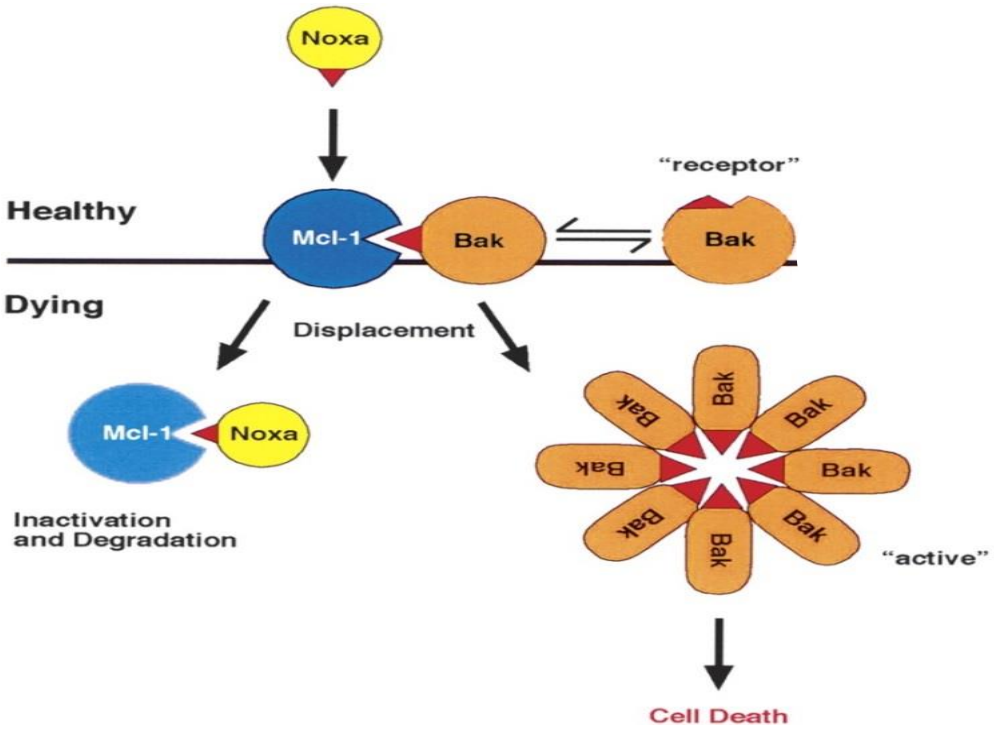


Figure 3: Induction of Noxa and its interaction with MCL-1 to release BAK from MCL-1 for oligomerization [21].

1.6 Cisplatin

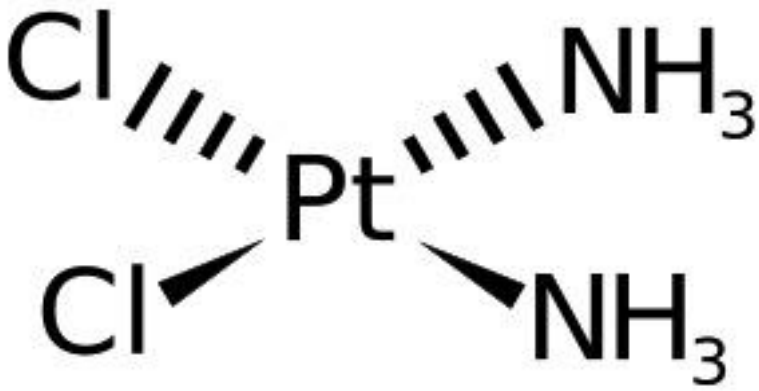
Cisplatin is a chemotherapeutic drug that is widely used to treat many different types of cancers including non-small cell lung cancer, bladder cancer, cervical cancer, ovarian cancer, head and neck cancer, and testicular cancer. It is often used to treat metastasized and advanced types of cancers that cannot be treated by other methods, such as radiation or surgery. Cisplatin works by interfering with the process of cell division,

inducing cancer cell death and slowing or stopping the spread of cancer. The history of cisplatin starts in 1844 when it was first created by Italian chemist Michele Peyrone. For a long time, it was known as Peyrone's chloride. In the mid-1900s, a biophysical chemist Barnett Rosenberg and his colleagues have demonstrated cisplatin's ability to inhibit cell division and reduce the size of solid tumors [9,10]. Despite its wide use to treat many different types of cancers, its complete mechanism as to how cisplatin induces cell death is not fully understood.

Cisplatin (cis-$PtCl_2(NH_3)_2$) consists of 11 atoms with platinum in the center (Figure 1) [11]. What we do know is that cisplatin works by nucleophilic substitution reaction. When cisplatin is intravenously injected, the chlorine atom gets replaced by a hydroxyl group and binds covalently to DNA in the cell (Figure 2) [12]. The platinum in the center is the key player to induce cell death in cancer cells, because this atom covalently binds to adjacent N-7 guanine to form 1,2 intrastrand crosslinks [13,14]. This crosslinking interaction results in many cellular responses including transcription inhibition, cell-cycle arrest, DNA repair and apoptosis (Figure 2) [12]. The most common form of death induced by cisplatin is through the BCL-2 family proteins to lead into apoptosis.

As a DNA damaging agent, cisplatin is often employed for the treatment of many different types of tumors, including HNSCC. However, its prolonged use often leads to the development of resistance [20]. It has been shown that a pro-apoptotic BH3-only protein, Noxa is transcriptionally induced by cisplatin. When Noxa is induced, it binds to and recruit MCL-1 at the outer membrane of the mitochondria, and release MCL-1 from its interaction with BAK and/or BAX. Recruited MCL-1 is subsequently phosphorylated at

Ser64/Thr70 by the formation of a complex with CDK2, leading to its proteasome-mediated degradation [20]. When BAK/MCL-1 interaction is released, activated BAK can now go through conformational change and oligomerize to form a pore at the outer membrane of the mitochondria, releasing cytochrome c into the cytosol for further caspase-9 activation.



http://www.scbt.com/datasheet-200896-cisplatin.html

Figure 4: The structural formula for cisplatin. The chemical name for cisplatin is *cis*-diamminedichloridoplatinum(II)

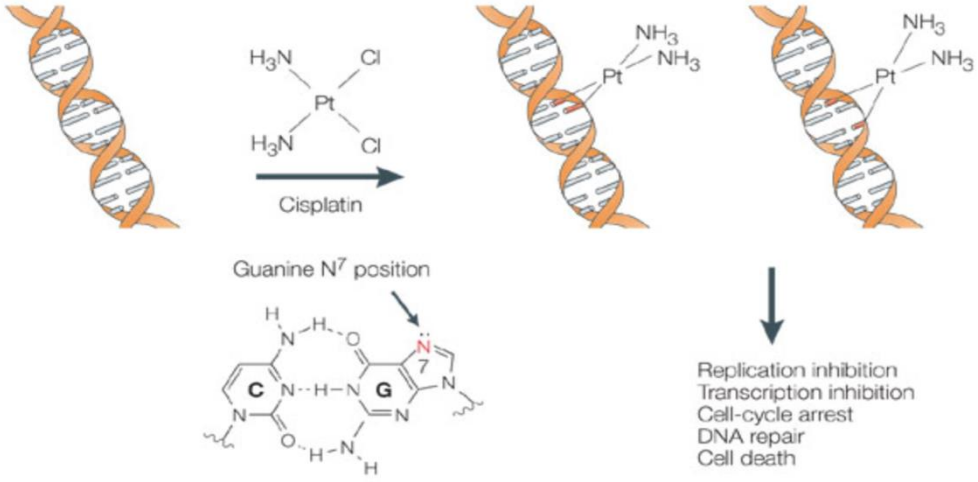


Figure 5: Molecular interaction of cisplatin**.** Cisplatin's cross-linking with DNA to inhibit replication and induce cell death [12].

1.7 Fenretinide

Fenretinide (N-4-hydroxyphenyl-retinamide, or 4-HPR) is a semisynthetic retinoid (Vitamin A) that was initially developed as a low-dose chemopreventative agent [www.cancernetwork.com]. It has a vitamin A activity and is well-known for its anti-tumor and differentiation-inducing activity *in vitro* and *in vivo*. While the mechanism of action of fenretinide-induced apoptosis is not fully understood, it has been reported that fenretinide binds to and activates retinoic acid receptors (RARs), thereby inducing cell differentiation and apoptosis in some tumor cell types. This agent also inhibits tumor growth by modulating angiogenesis-associated growth factors and their receptors and exhibits retinoid receptor-independent apoptotic properties [www.cancer.gov]. Studies have shown that in certain tumor types, such as neuroblastoma and melanoma, fenretinide induces apoptosis

through endoplasmic reticulum (ER) stress, which is mediated by the eukaryotic initiation factor 2alpha (eIF2alpha)-ATF4 signaling pathway [23]. Increased expression of eIF2alpha and ATF4 correlated with induction of the BH3-only pro-apoptotic protein Noxa, followed by inhibition of multi-domain anti-apoptotic protein MCL-1.

HNSCC has a high incidence of recurrence and associated second primary malignancy. Fenretinide has been shown to be effective as both a chemopreventive and chemotherapeutic agent for HNSCC, but often with treatment-limiting toxicity. Fenretinide has significant anti-proliferative activity against a number of animal and human malignancies and has been used in clinical trials as a chemopreventive agent in patients with breast and prostate cancer and oral leukoplakia [24]. Through many research experiments, fenretinide has been shown promise for treating many different types of tumors, including HNSCC. The ability of fenretinide to enhance production of reactive oxygen species (ROS) leading to apoptosis has been suggested as a possible mechanism [29]. Rac was activated in human HNSCC cells as early as five minutes following fenretinide exposure. Moreover, inhibition of Rac activity or silencing of its expression by RNA interference decreased the ROS generation in human head and neck, lung, and cervical cancer cells [29]. ROS is also known to be able to induce an ER stress response, which can contribute to apoptosis, but may also antagonize it [30]. It has been reported that combination of ABT-737, an inhibitor of BCL-X_L, BCL-2, and BCL-W, and fenretinide have synergistically decreased cell viability and caused death in multiple melanoma cell lines, but not in normal melanocytes [25]. The combination of ABT-737 and fenretinide have increased the Noxa expression and caspase-dependent MCL-1 degradation [25].

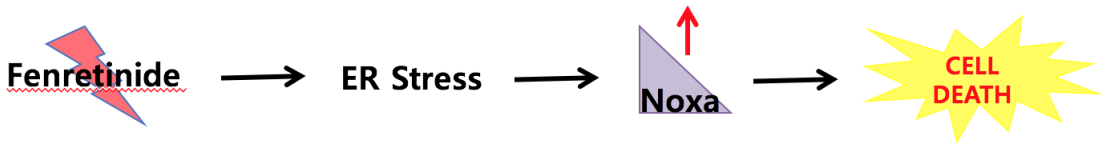
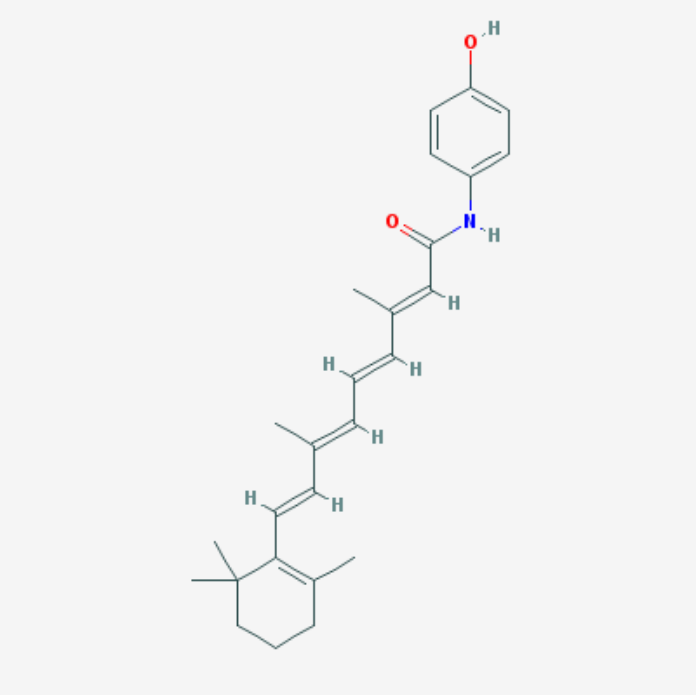


Figure 6: ER-stress mediated cell death. ER stress leads to apoptotic cell death at transcriptional, post-transcriptional, translational, and post-translational levels. Pro-apoptotic proteins, such as Noxa is induced as a result.



https://pubchem.ncbi.nlm.nih.gov/compound/fenretinide#section=Top

Figure 7: Structural formula of fenretinide.

1.8 ABT-263 (navitoclax)

The BCL-2 family comprised of two broad categories of pro-survival (BCL-2, BCL-X_L, BCL-W, MCL-1, and A1) and pro-apoptotic (Noxa, BAX, BAK, BID, BIM, and BAD) proteins [27]. The balance between these proteins determines whether a cell lives or dies. The overexpression of pro-survival BCL-2 family members provides a mean by which cancer cells can overcome the continual assault of cellular stresses that would cause normal cells to undergo apoptosis. Their overexpression has also been shown to correlate with poor prognosis and resistance to therapy [27]. For example, overexpression of BCL-2 has been shown to confer resistance to cytotoxic agents in lung cancer, whereas both BCL-2 and BCL-X_L are associated with the emergence of androgen independence and chemoresistance in prostate cancer [27].

ABT-263, also known as navitoclax, is an orally bioavailable, synthetic small-molecule antagonist of a subset of the BCL-2 family of proteins with potential antineoplastic activity [26]. It selectively binds to anti-apoptotic proteins BCL-2, BCL-X_L, and BCL-W, and prevents their binding to the apoptotic effectors BAX and BAK proteins, which triggers apoptosis in tumor cells overexpressing BCL-2, BCL-X_L, and BCL-W. In human tumor cells, ABT-263 is known to induce BAX translocation, releasing cytochrome c into the cytosol from the mitochondria, and subsequent caspase-dependent apoptosis. While ABT-263 exhibits single-agent activity in tumors dependent on BCL-2 and BCL-X_L for survival, the expression of MCL-1, the main contributor to the caspase-dependent apoptotic pathway has been shown to confer resistance to ABT-263. Furthermore, in some xenograft models of aggressive lymphoma and in phase I/II clinical studies in patients of

lung cancer, single-agent ABT-263 just exhibited modest or limited efficacy, with dose-dependent thrombocytopenia induced by targeting BCL-X_L in megakaryocytes [28]. To solve this limitation, recent studies are more focusing on combining the ABT-263 with other chemotherapeutic drugs. For this reason, we can not only minimize the side effects by combining ABT-263 along with Noxa-inducers (cisplatin or fenretinide), but also to simultaneously block multi-domain anti-apoptotic proteins (BCL-2, BCL-X_L, and MCL-1) to efficiently increase the amount of apoptosis in HNSCC.

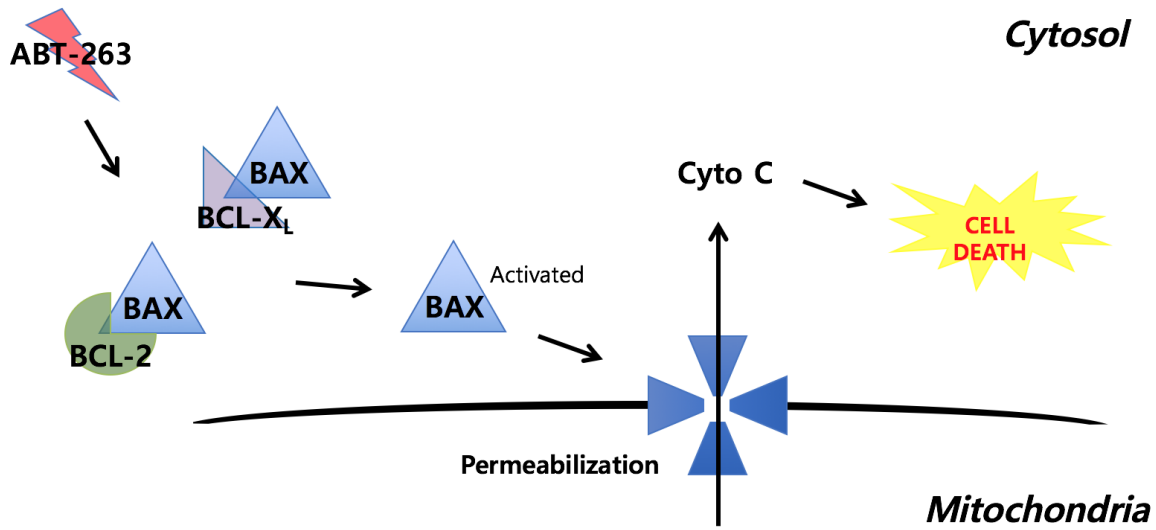
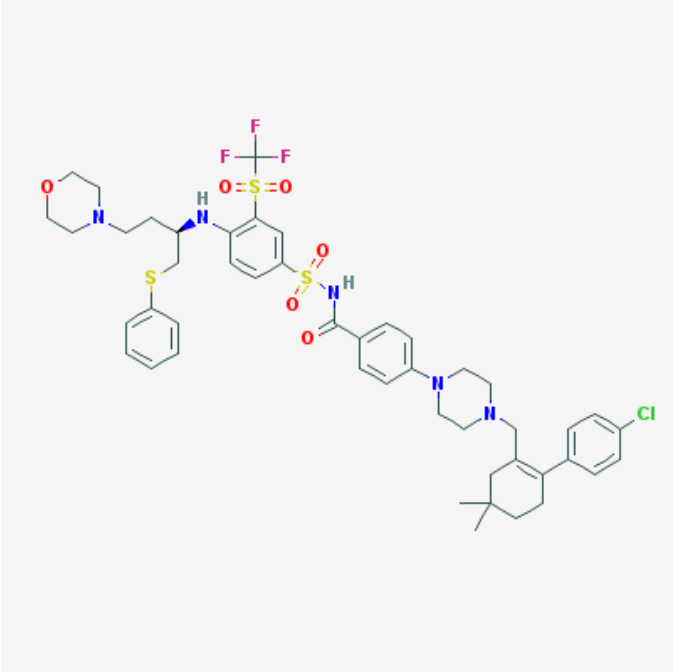


Figure 8: Translocation of BAX to the outer membrane of mitochondria. BAX translocation leads to release of cytochrome c into the cytosol for caspase-dependent apoptotic cell death.



https://pubchem.ncbi.nlm.nih.gov/compound/Navitoclax#section=Top

Figure 9: Structural formula of ABT-263.

1.9 p53

p53 was first identified in 1979 by Arnold Levine, David Lane, and William Old. Although it was first presumed to be an oncogene, its character as a tumor suppressor gene was revealed in 1989 [www.bioinformatics.org]. It is very important for cells in multicellular organisms to suppress cancer. p53 has been described as "the guardian of the genome", referring to its role in encoding for proteins that regulate the cell cycle and hence functions as a tumor suppressor. Defective p53 can allow abnormal cells to proliferate, resulting in cancer. As many as 50% of all human tumors contain p53 mutants. p53 is often

activated by stresses, such as ultraviolet, x-rays, chemotherapeutic drugs, and DNA synthesis inhibitors. Kinases, such as Chk1, Chk2, and ATM phosphorylate p53 protein to prevent from degradation. High concentration of p53 stops the cell cycle at G1/S and induces DNA repair proteins to fix the damage and prevent replication of the damaged DNA. When DNA damage is repaired, phosphorylation by kinases that protect the p53 degradation disappear and the cell cycle resumes its activity to replicate the DNA in the S phase. If DNA damage cannot be repaired, p53 acts as a transcription factor to induce the expressions of several pro-apoptotic target genes, such as Noxa and Puma for the apoptotic cell death. The cellular concentration of p53 must be tightly regulated. In normal cells, p53 protein level is low and this is because p53 is constantly being degraded by the major regulator protein called MDM2 (in human). This protein binds to the N-terminus of p53 and triggers polyubiquitylation and proteasome-mediated destruction.

HYPOTHESIS

Cisplatin induces anti-cancer effects through the induction of BCL-2 family-dependent mitochondrial apoptosis. One of the BCL-2 family proteins that are induced is Noxa. This protein selectively binds to anti-apoptotic MCL-1 to inactivate its function and release BAK to cause apoptosis. Similarly, fenretinide has been shown to induce Noxa through ER stress and inactivate the function of anti-apoptotic protein MCL-1. On the other hand, ABT-263 selectively and directly binds to the apoptosis suppressor proteins, such as BCL-2 and BCL-$X_{L,}$ but not MCL-1. This direct binding prevents anti-apoptotic proteins from binding to the multi-domain pro-apoptotic proteins BAK and/or BAX. **We hypothesize that combining the Noxa-inducing drugs (cisplatin or fenretinide) with ABT-263 can efficiently induce BAX and/or BAK activation and significantly increase cell death in HNSCC cells by simultaneously inhibiting the activity of MCL-1, BCL-2, and BCL-X_{L}.**

SPECIFIC AIMS

3.1 Determine how much apoptosis can increase in HNSCC cells by treating with both cisplatin or fenretinide along with ABT-263, compared to single treatments.

The toxicity level of cisplatin, fenretinide, ABT-263, and combination was measured. Cell death was determined by Western blot analyses and Annexin V-propidium iodide (PI) staining, followed by FACS analyses.

3.2 Determine the molecular mechanisms of Noxa-induced cell death in HNSCC cells.

The lentiviral short-hairpin Noxa (shNoxa) expressing constructs were introduced to HNSCC cells to infect and knock-down the expression of the Noxa to determine the involvement of Noxa in cisplatin, feretinide, ABT-263, and combination-induced cell death. The cell death will be determined by Western blot analyses and Annexin V-PI staining, followed by FACS analyses.

MATERIALS AND METHODS

4.1 Cell lines and cell culture

HN12, HN22, HN8, HN30, and HN31 head and neck squamous cell carcinoma (HNSCC) cells were provided by Dr. Andrew Yeudall (Augusta University, GA), UMSCC1 cells were provided by Dr. Yue Sun (Virginia Commonwealth University). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with addition of 10% heat-inactivated fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and 5% 100 μg/mL penicillin G/streptomycin (Invitrogen) at 37°C in a humidified, 5% CO_2 incubator. 293T cells were purchased from the American Type Culture Collection (Manassas, VA). HN30/sh-Noxa, HN30 shC, UMSCC1/sh-Noxa, and UMSCC1 shC cells were maintained with 2 μg/mL of puromycin (InvivoGen, San Diego, CA) for selection.

4.2 Plasmid transfection and lentivirus infection

The lentiviral short-hairpin RNA (shRNA) expressing constructs were purchased from Open Biosystems (Huntsville, AL) or Sigma-Aldrich (St. Louis, MO). The constructs were transfected into 293T packaging cells along with the packaging plasmid (Addgene; Cambridge, MA) and lentivirus-containing supernatants were collected. A cell line of interest was seeded in a 6-well plate and the plate containing the media (DMEM) was removed on the following day. Subsequently, 1 mL of DMEM and 1 mL of collected supernatant containing the lentiviruses were added, and further treated with 2 μL of

polyburene. It was then spun down at 2000 rpm for 1 hour in the centrifuge to facilitate the binding process of viruses to the cells.

4.3 Chemicals and antibodies

Cisplatin (sc-200896) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for BIM, Cleaved PARP (Asp214), GAPDH (D16H11), Cleaved Caspase -3 (Asp175), HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG were from Cell Signaling Technology (Beverly, MA); MCL-1 (ADI-AAP-240-F) from Enzo Life Sciences (Farmingdale, NY); p53 (sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA); Noxa (114C307.1) from Thermo Fisher Scientific (Waltham, MA); ECL 2 Western blotting substrate (80196) was purchased from Thermo Scientific (Rockford, IL). Annexin V-FITC was purchased from BD Biosciences (California, USA), and propidium-iodide from Sigma Aldrich Inc. (St. Louis, MO).

4.4 Western blot

To perform Western blots, whole HNSCC cell lysates were prepared with CHAPS lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1:200 ratio of protease inhibitor cocktail (Sigma Aldrich), and 1:100 ratio of phosphatase inhibitor cocktails 2 and 3 (Sigma)]. Protein concentrations from the lysates were measured by spectrophotometric analysis with the Bradford method (Bio-rad, Hercules, CA), and equal amounts of samples were loaded into SDS-polyacrylamide gels. The gel was electrophoresed at 180 volts for

approximately 45 minutes and the proteins were transferred onto a nitrocellulose membrane (Fischer Scientific, Pittsburgh, PA) with 100 volts for one hour. The nitrocellulose membrane was then blocked with a blotting solution [5% skim milk in PBST (1 x PBS with 0.1% Tween-20)] for 20 minutes, and specific primary antibodies were incubated with the membrane for overnight at 4°C. The incubated membrane was washed with PBST for at least five minutes for three times and then either HRP-linked anti-mouse IgG or anti-rabbit IgG antibodies were incubated with the membranes for one hour at room temperature. The membranes were washed three times with PBST for five minutes. The membrane was then developed using Pierce ECL- 2 Western Blotting Substrate (Thermo Fisher Scientific).

4.5 FACS analysis

To perform fluorescence-activated cell sorting (FACS) analysis, cell lines of interest were seeded in a 12-well plates and treated with cisplatin (20 μM), fenretinide (10 μM), ABT-263 (1 μM), and combined drugs. Cells were harvested at 24 or 48-hour time points and was spun down in the centrifuge for one minute at 7000 rpm. Then, the cell pellets in each tube were suspended in 1 ml of cold 1x PBS. PBS-washed cells were then spun again in the centrifuge for one minute at 7000 rpm, followed by aspiration of the supernatant. Then, 100 μL of binding buffer was added into each tube and transferred to a 5 ml polystylene tube. Total 13 μL of Annexin V (3 μL)-propidium iodide (10 μL) was added into each tube, and was left in the dark for 15 minutes to avoid bleaching dyes. Finally, additional 400 μL of binding buffer was added into each tube and was analyzed through the FACS Caliber (BD Biosciences).

4.6 Cell toxicity (WST-1) assay

HNSCC cells were seeded in triplicate in microtiter plates (96 wells) with $1x10^4$ cells per well in 150 μL medium. On the following day, cells were treated with different concentration of cisplatin and 48 hours later, 2 μL of WST-1 reagent was added to the cells. The WST-1 assay is based on tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3- benzene disulphonate). It is used to determine the cytotoxicity of cisplatin by measuring the absorbance of the samples with a microplate enzyme-linked immunosorbent assay (ELISA) reader (Promega Life Sciences).

4.7 IC_{50}

IC_{50} (half maximal inhibitory concentration) is a term that represents an amount of specific substance (inhibitor) needed to inhibit 50 percent of cell's biological or biochemical mechanisms, i.e. an enzyme, receptors or microorganism. IC_{50} for cisplatin cytotoxicity was calculated for each HNSCC cell line by obtaining quantitative measurements from the microplate ELISA 450nm reader. The measurements were entered in Excel sheets to calculate the IC_{50}.

4.8 Statistics

The mean, or average, represented by bar graphs is calculated by finding the sum of the study data and dividing it by the total number of data. Error bars indicated by a line through the graph represent the error of the corresponding study (Mean ± S.D.). The standard deviation (S.D.) is used to quantify the amount of variation of a set of data values. Errors

were calculated by taking the difference between the largest and smallest values of our collected data. P-values were also calculated through t-test in order to quantify the idea of statistical significance of our evidence. P-values < 0.05 suggest that our results are statistically significant.

RESULTS

5.1 HNSCC cell lines and their cisplatin sensitivity, IC_{50}

Table 1 contains the list of head and neck squamous cell carcinoma cell lines that were used in this study and their primary origin as well as main characteristics [34]. Cell lines that are harvested from the lymph node are post-metastatic. The *p53* gene in HN8 is deleted, HN30 and UMSCC1 harbors the wild-type *p53*, and HN31 harbors a mutation within the *p53* gene, but the gene still retains some function [20,31,32].

TABLE 1. HNSCC Cell Lines

Patient	Cell Line	Origin	p53 Status	IC_{50} (µM) Cisplatin
A	**HN8**	Lymph node	Deleted	50
B	**HN30**	Pharynx	Wild-type	21
	HN31	Lymph node	Mutated	20
C	**UMSCC1**	Floor of mouth	Wild-type	15

IC_{50} of the cell lines were determined by the WST-1 (tetrazolium salt) assay. When cells are metabolically active, WST-1 is cleaved to form a soluble formazan dye, which can be quantified by a scanning multi-well spectrophotometer at 450 nm (Figure 8) [33].

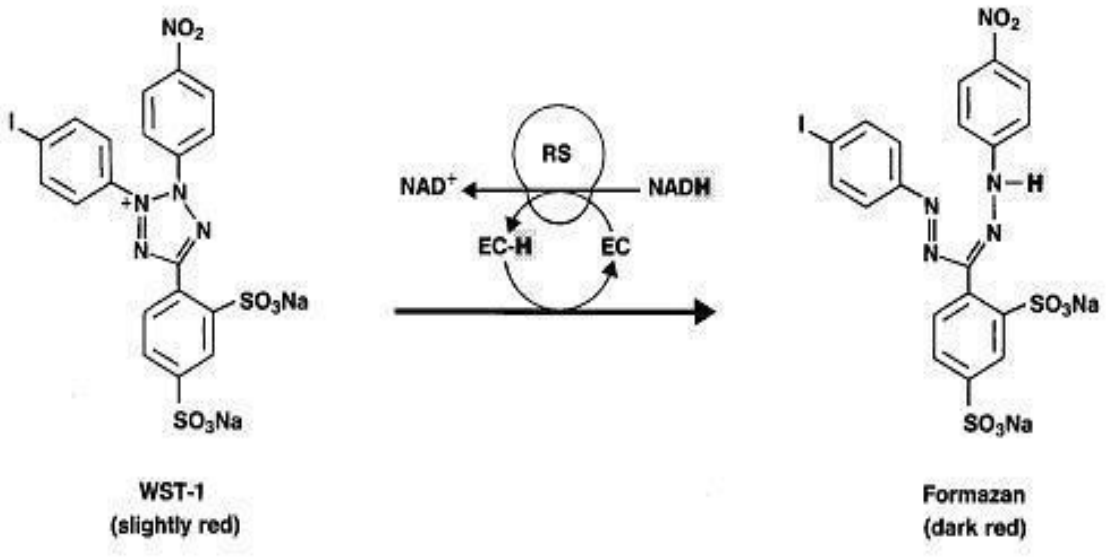
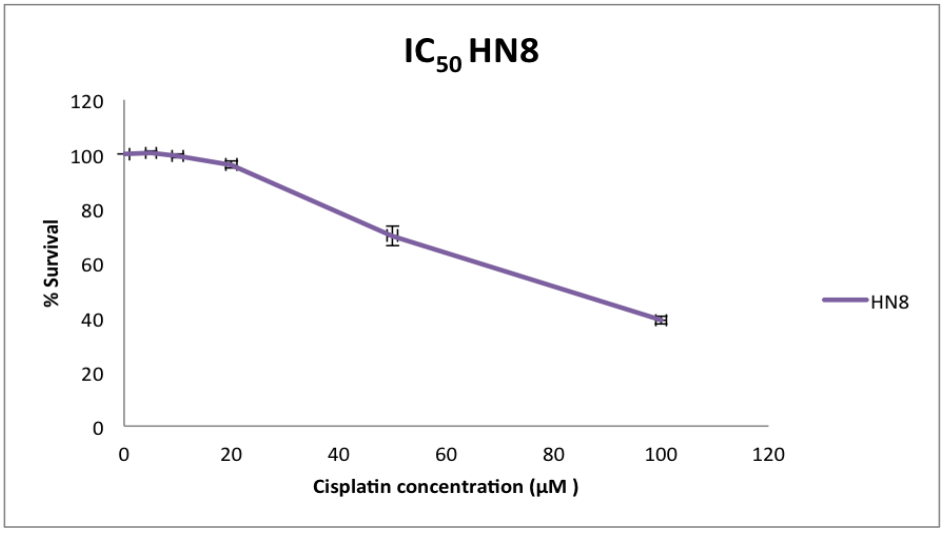


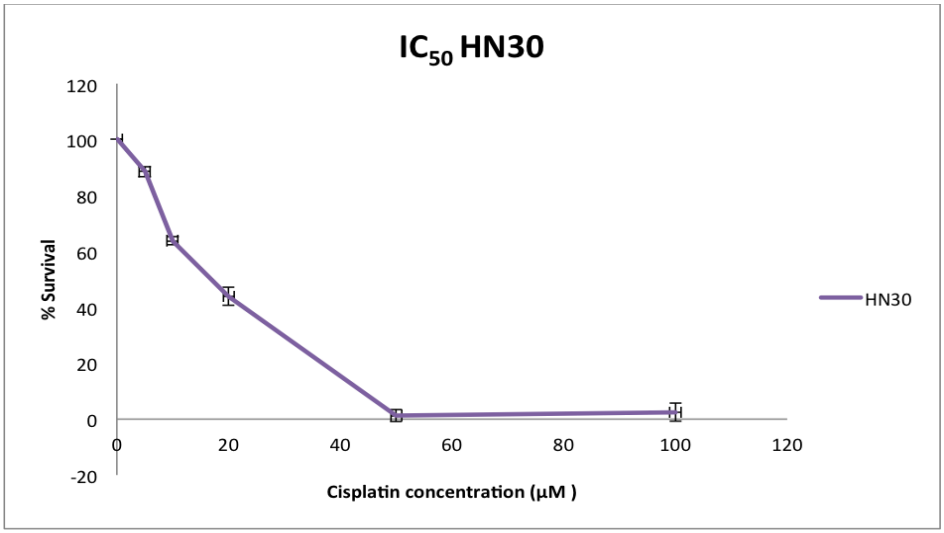
Figure 10: Cleavage of WST-1 (tetrazolium salt) to formazan. (EC: electron coupling reagent, RS: mitochondrial succinate-tetrazolium-reductase system)[33].

We found that p53 wild-type cell UMSCC1 had the lowest IC_{50} of 15 µM, while p53-deleted HN8 had the highest IC_{50} of 50 µM. HN30 that has wild-type p53 and HN31 with mutant p53 had IC_{50} of 21 µM and 20 µM, respectively.

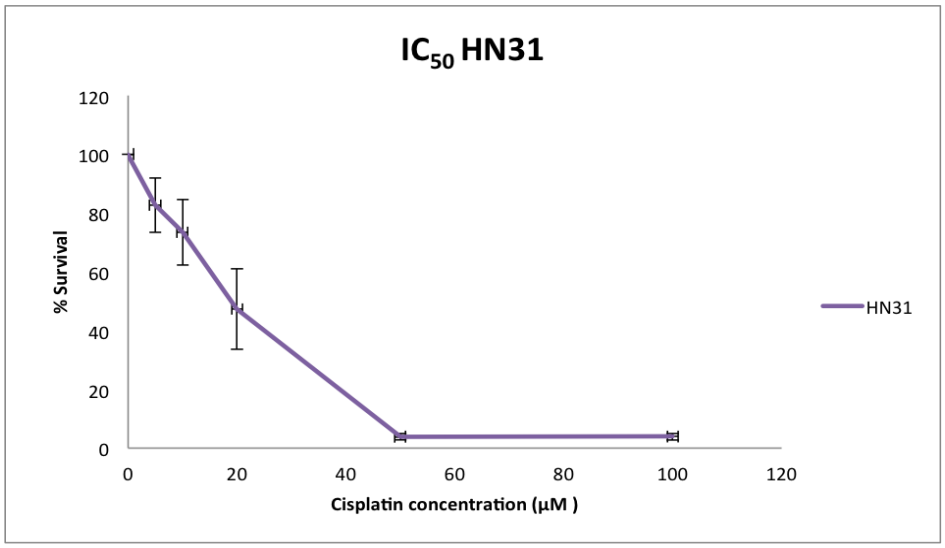
A)


IC50 HN8
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN8

B)


IC50 HN30
120
100
80
60
40
20
0
-20
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN30

C)



D)

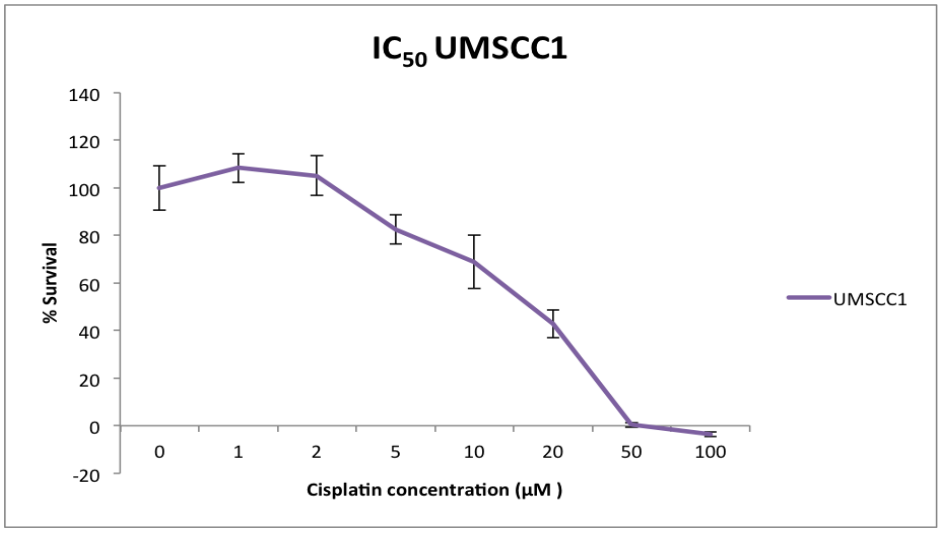


Figure 11 (A-D): IC_{50} of cisplatin treatment in HNSCC cell lines. HNSCC cell lines were treated with various concentrations of cisplatin for 48 hours. Percentage of survival was determined by the WST-1 assay [34].

5.2 Noxa-inducers combined with ABT-263 show dramatic increase in apoptotic cell death in HNSCC cells.

To examine whether combination-induced apoptotic cell death is dramatically increased in HN8, HN30, HN31, and UMSCC1, each cell line was seeded in 6-well plates and treated with DMSO for control, cisplatin (20 μM), fenretinide (10 μM), ABT-263 (1 μM), cisplatin with ABT-263, and fenretinide with ABT-263. Cells were harvested at 16-hour time point and probed with cleaved-PARP, MCL-1, BIM, and Noxa. PARP is a substrate that is cleaved off when caspase-3 is activated during caspase-dependent apoptosis, and therefore is a strong indication of apoptotic event.

HN8 cell line has a noticeable increase in cleaved-PARP when treated with fenretinide and ABT-263 together compared to each treatment alone, indicating the synergistic cell death activity with these two chemotherapeutic drugs. The expression level of Noxa is also consistent with the amount of cleaved-PARP, suggesting that fenretinide or fenretinide with ABT-263-induced apoptotic cell death is largely depending on Noxa. However, the effect of cisplatin with ABT-263-induced apoptotic cell death seems additive as combination-induced cell death is not significantly increased compared to single treatment. Our result observed in Western blot analysis is also consistent with the cell death observed through FACS analysis.

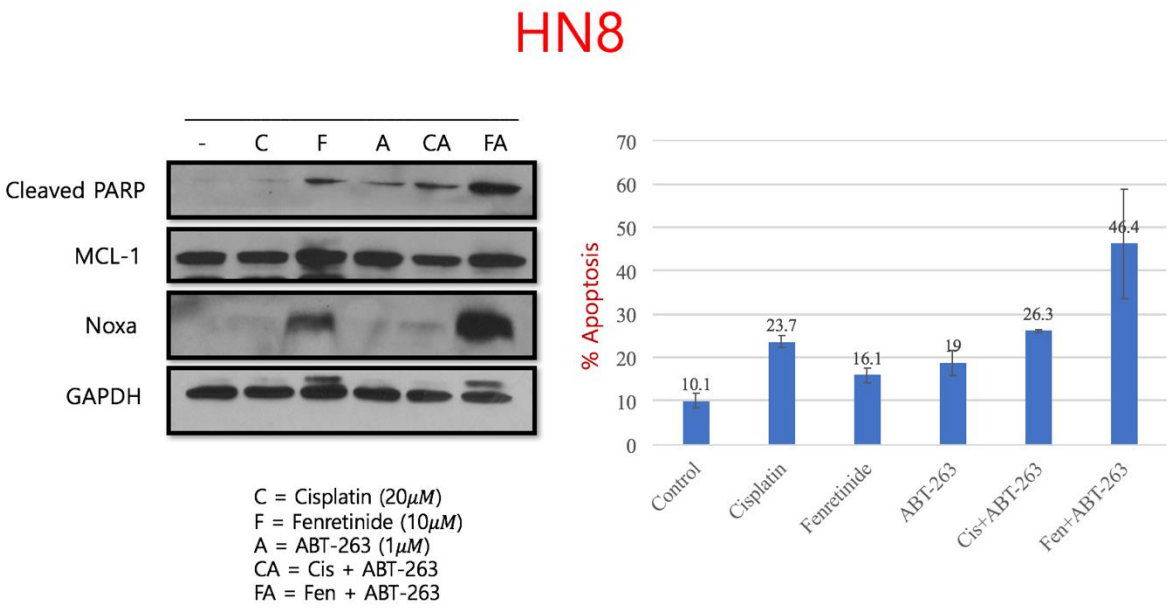


Figure 12: HN8 is noticeably responsive to fenretinide + ABT-263 combination treatment. Left) HN8 cells were treated with indicated drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies. GAPDH

was used to confirm the equal loading of total lysates. **Right)** HN8 cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

HN30 is one of the cell lines we observed the most synergistic increase of apoptotic cell death. We observed a significant increase in cleaved-PARP as a result of combination-induced cell death, both in CA (cisplatin + ABT-263) and FA (fenretinide + ABT-263). The expression level of Noxa has noticeably increased in combined treatment, which well-correlated with the cleaved-PARP. MCL-1 is also greatly reduced as a result of induction of Noxa under fenretinide + ABT-263-induced apoptosis.

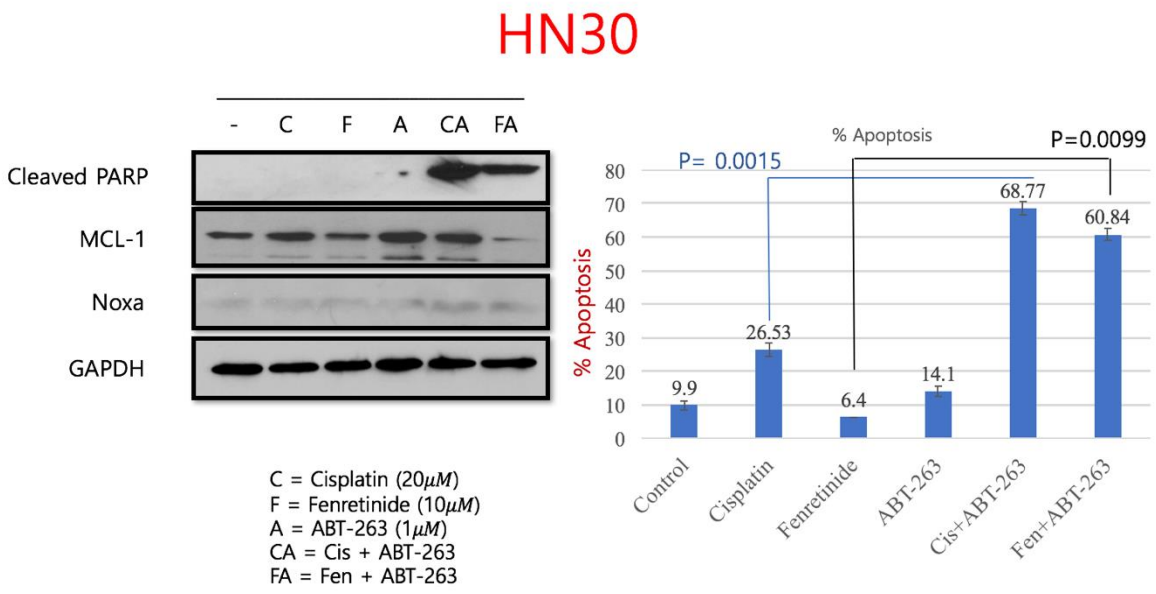


Figure 13: HN30 is largely responsive to both types of combination-induced apoptosis.

Left) HN30 HNSCC cells were treated with indicated drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies. GAPDH was used to confirm the equal loading of total lysates. **Right)** HN30 cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

The HN31 cell line is established from the same patient, although p53 is mutated. However, this cell line was more responsive to cisplatin + ABT-263-induced treatment. The level of MCL-1 is inversely correlated with the expression level of Noxa. Cisplatin and ABT-263 together resulted in most apoptosis, which was indicated by cleaved-PARP. Single treatment of cisplatin seems more effective in inducing apoptosis than fenretinide + ABT-263 combined treatment. Our result from the Western blot analysis is also confirmed through FACS analysis and the amount of total apoptosis is also well-correlated.

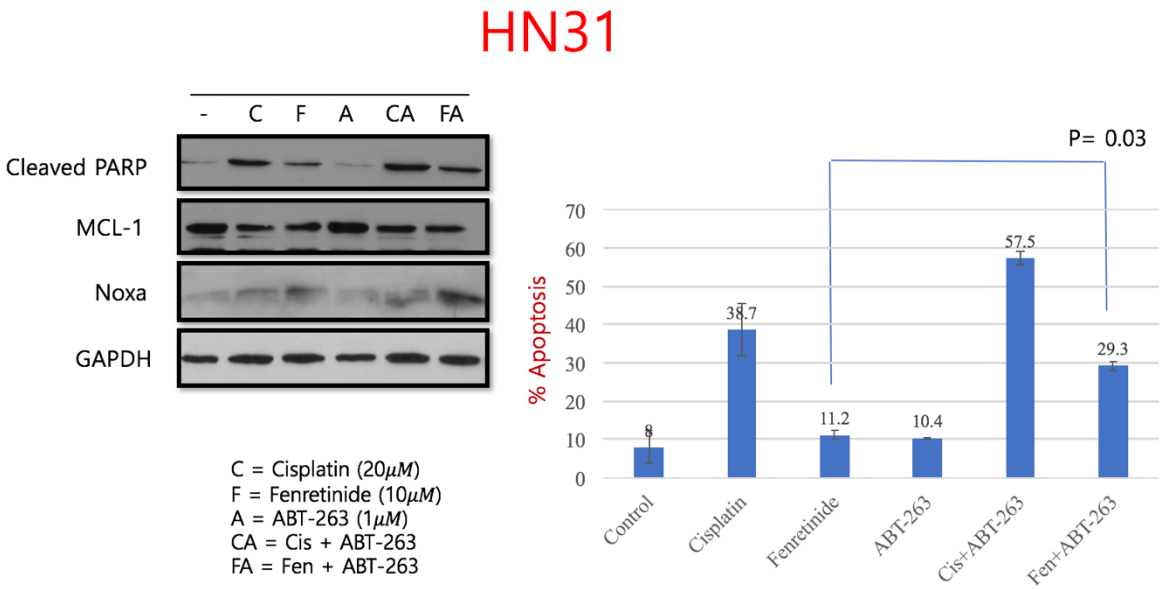


Figure 14: Combination-induced cell death has significantly increased apoptosis. **Left)** HN31 HNSCC cells were treated with indicated drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies. GAPDH was used to confirm the equal loading of total lysates. **Right)** HN31 cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

UMSCC1 also showed similar response as HN31 in terms of cleaved-PARP. It is well-responded to cisplatin + ABT-263 combination treatment. Cisplatin + ABT-263 combination results in the most apoptosis, followed by cisplatin alone, and fenretinide + ABT-263 combined treatment. The expression of Noxa is strongly induced by combined treatments, but also by cisplatin alone. We also observed that BIM, another BH3-only pro-apoptotic protein, has greatly reduced by combination-induced cell death. Our result from the Western analysis is further confirmed through FACS analysis.

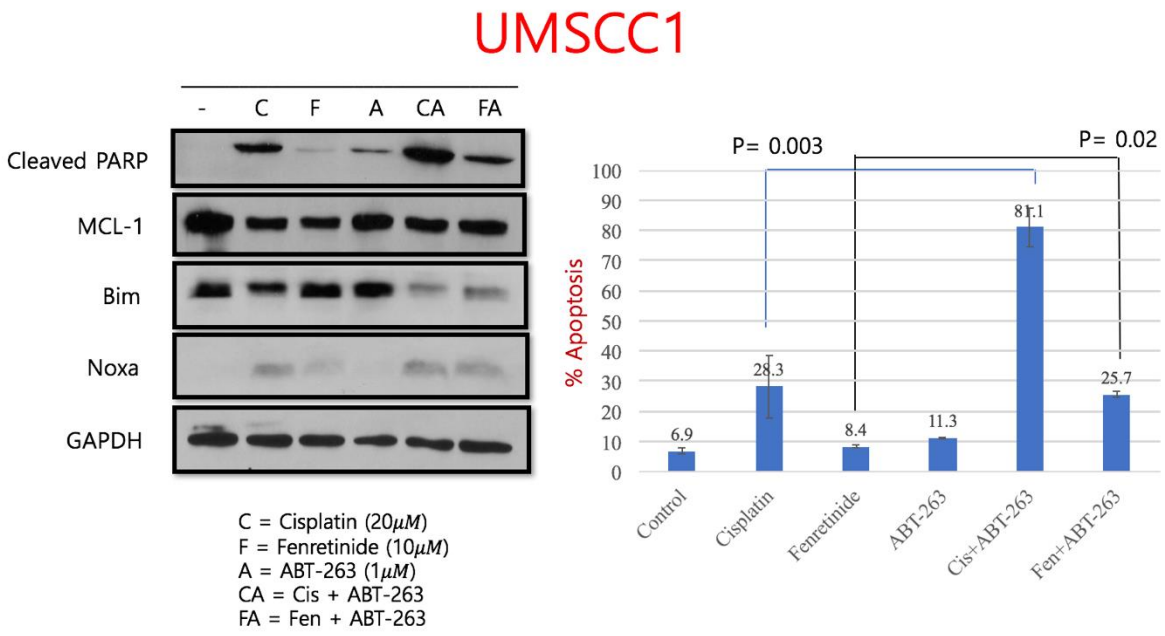


Figure 15: Cisplatin + ABT-263-induced treatment significantly increased the apoptotic cell death.

Left) UMSCC1 cells were treated with indicated drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies. GAPDH was used to

confirm the equal loading of total lysates. **Right)** UMSCC1 cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

5.3 The contribution of Noxa in combination-induced cell death.

To examine if combination treatment induces Noxa to cause cell death, we introduced short-hairpin RNA (shRNA) for Noxa to downregulate its expression. As control, we introduced non-targeting shRNA. Both sh-control and sh-Noxa expressing HNSCC cells were treated similarly as the previous experiments.

As shown in Figure 16 and 17, a clear reduction of apoptosis with down-regulation of Noxa was observed in cisplatin + ABT-263 and fenretinide + ABT-263. This reduction suggests that Noxa is contributing to apoptosis in these treatments. Knock-down of Noxa expression is also leading to greater amount of MCL-1. UMSCC1 in Figure 18 also shows a clear reduction in cleaved-PARP with decreased expression of Noxa. Both combined and single treatments are largely depending on the expression of Noxa, however, single cisplatin treatment is not affected by downregulation of Noxa, suggesting that its cell death is depending on other BH3-only pro-apoptotic proteins. Our result from the Western

analysis is further confirmed through FACS analysis, and the total amount of apoptosis was consistent.

Puma is another p53 target gene. We decided to examine whether BH3-only protein Puma is contributing to apoptosis. We observed that Puma is strongly induced under the cisplatin single treatment, whereas in cisplatin and ABT-263 combined treatment, Noxa is largely induced.

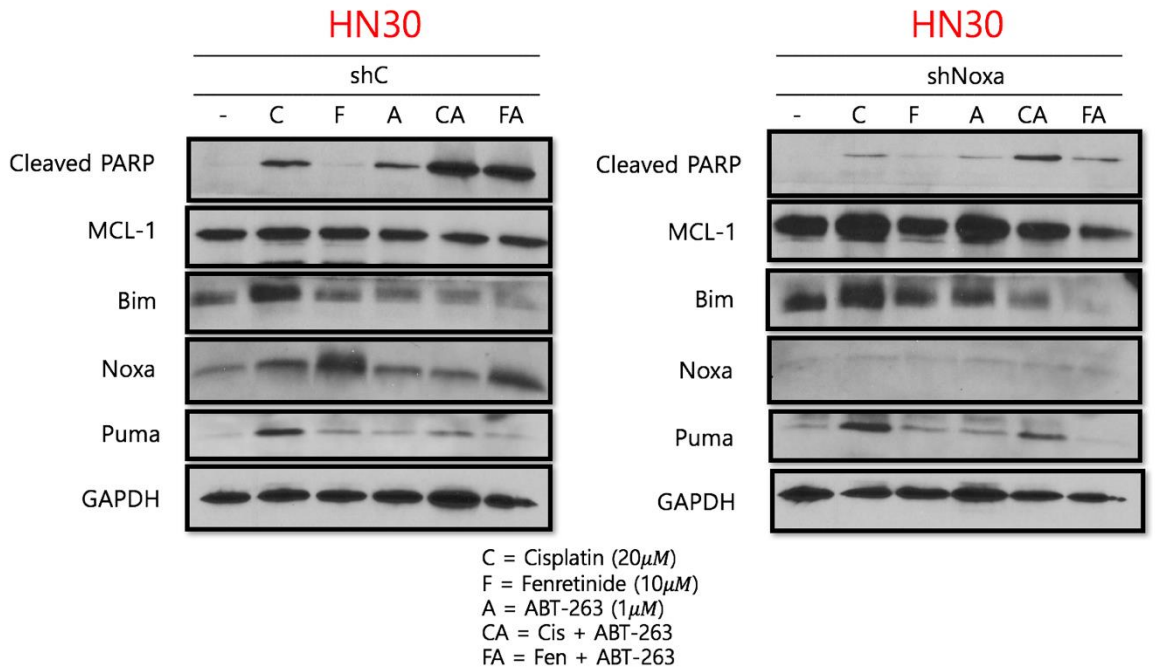


Figure 16: Noxa plays a critical role in the induction of apoptotic cell death in combined treatments in HN30. The lentiviruses encoding short-hairpin Noxa (shNoxa) or scrambled control (shC) were infected to HN30. Then the cells were treated with indicated

drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies.

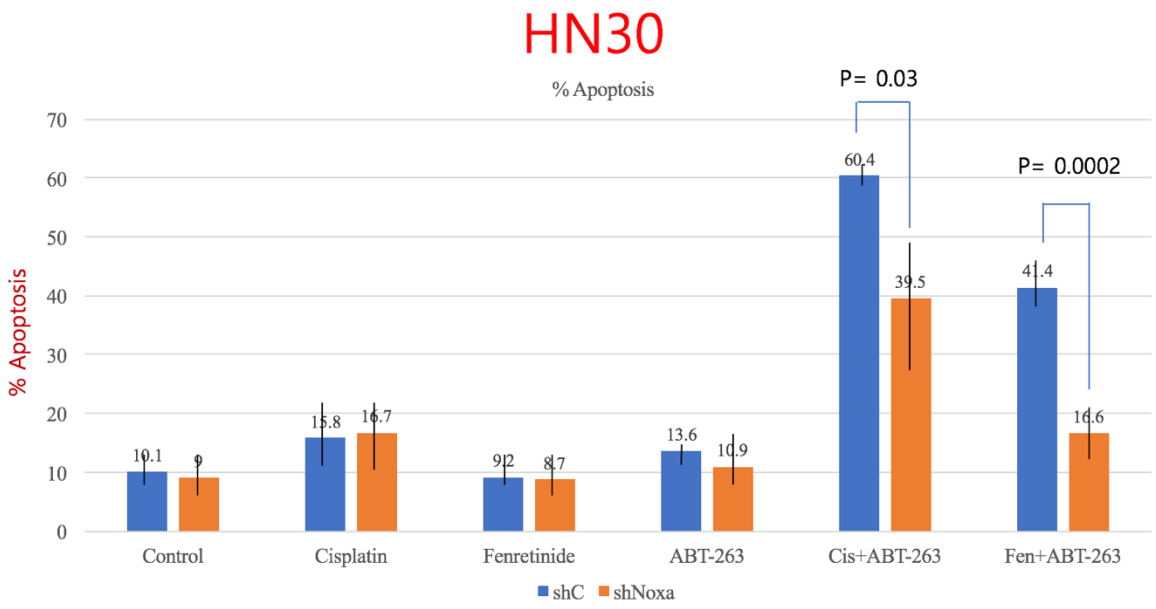


Figure 17: Combination-induced apoptotic cell death in HN30 is depending on the expression of Noxa. The lentiviruses encoding short-hairpin Noxa (shNoxa) or scrambled control (shC) were infected to HN30. Then cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

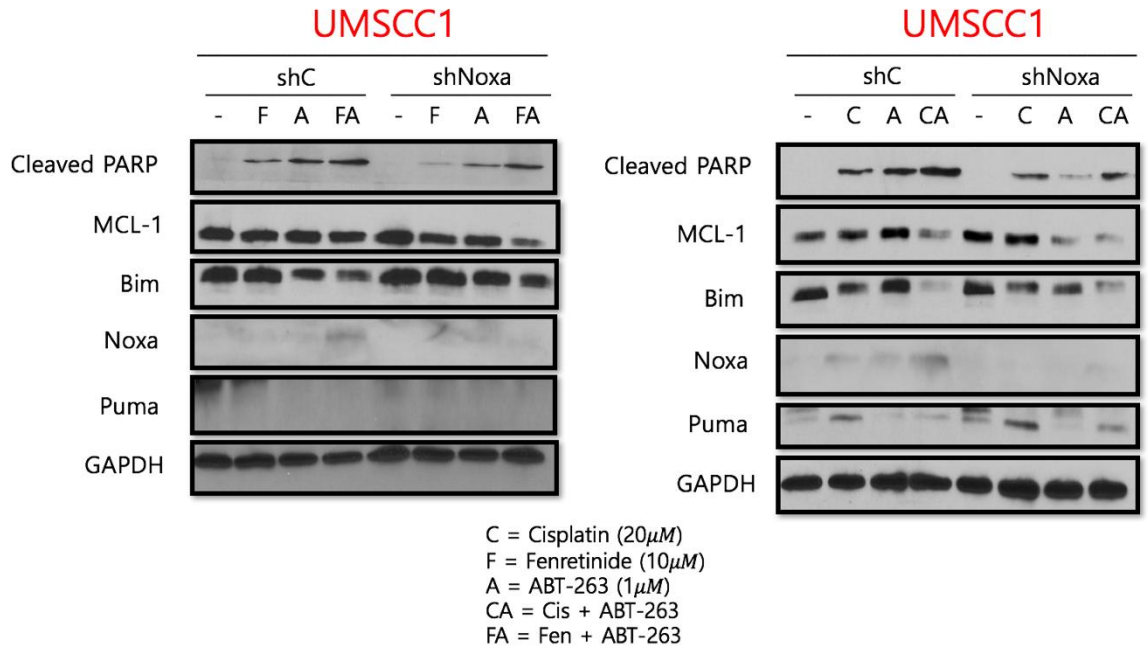


Figure 18: Noxa plays a partial role in induction of apoptotic cell death in fenretinide + ABT-263 combination treatment. The lentiviruses encoding short-hairpin Noxa (shNoxa) or scrambled control (shC) were infected to UMSCC1. Then the cells were treated with indicated drugs for 16 hours and equal amounts of samples were analyzed by Western blots using indicated antibodies.

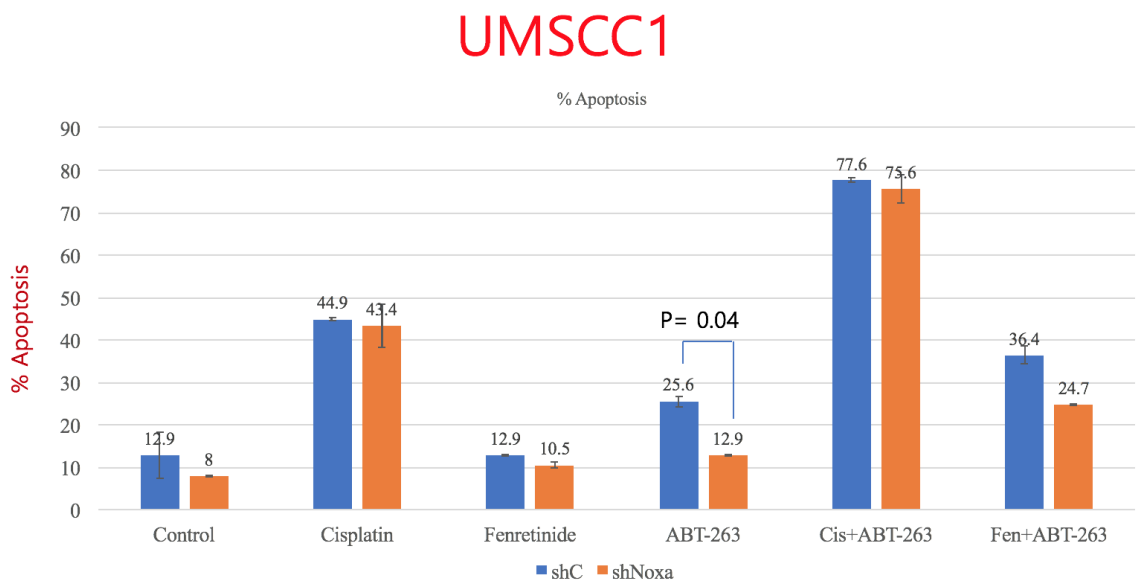


Figure 19: Fenretinide + ABT-263-induced apoptotic cell death in UMSCC1 is depending on the expression of Noxa. The lentiviruses encoding short-hairpin Noxa (shNoxa) or scrambled control (shC) were infected to UMSCC1. Then cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

5.4 The contribution of p53 in combination-induced cell death.

Cisplatin treatment of HNSCC cells results in DNA damage, which then activates p53 to prevent the replication of damaged DNA. If this damage is irreparable, p53 will act as a transcription factor to induce the expressions of several pro-apoptotic target genes. One of these proteins induced by activation of p53 is BH3-only pro-apoptotic protein Noxa. To examine whether p53 induces the expression of Noxa in combination treatments and further lead to apoptotic cell death, we introduced short-hairpin RNA (shRNA) for p53 to downregulate its expression. As control, we introduced non-targeting shRNA. Both sh-control and sh-p53 expressing HNSCC cells were treated similarly as the previous experiments. Figure 20 shows a clear reduction in cleaved-PARP in cisplatin-induced and ABT-263-induced cell death with decreased expression of p53. The expression levels of Noxa and p53 also correlates. However, we observed that decreased expression of p53 has no effect on cleaved-PARP under the fenretinide + ABT-263 (FA) treatment. On the other hand, FA combination-induced apoptotic cell death in UMSCC1 (Figure 21) was shown under the FACS analysis when p53 is down-regulated.

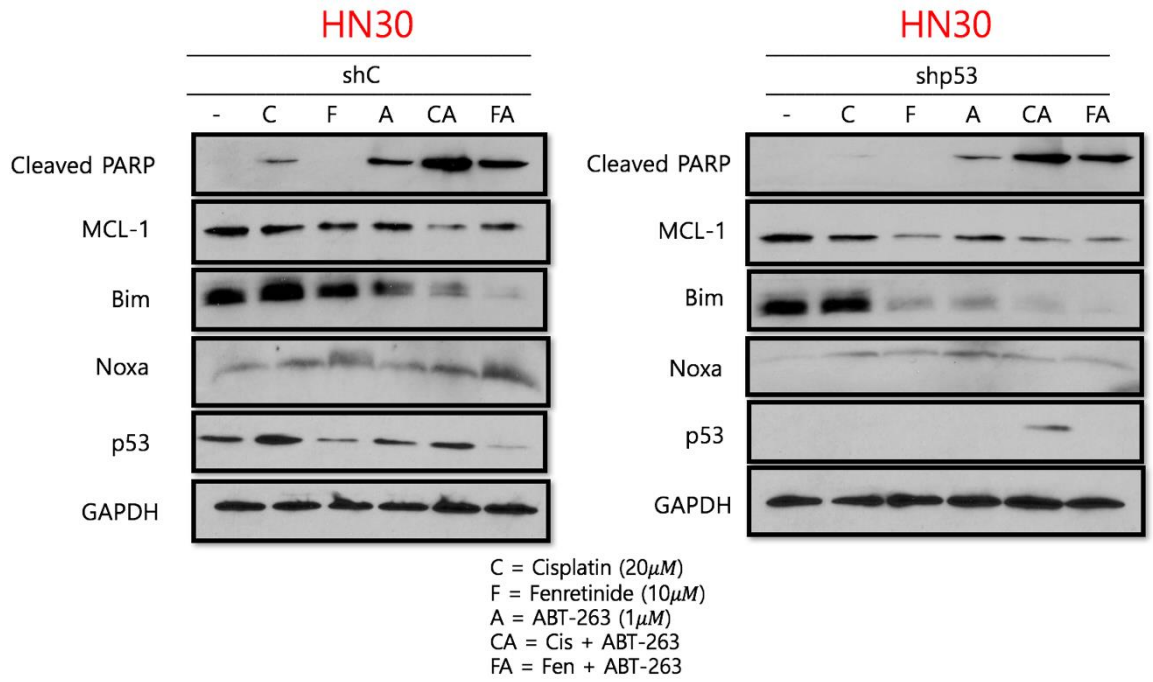


Figure 20: p53 is involved in fenretinide + ABT-263-induced cell death. The lentiviruses encoding short-hairpin p53 (shp53) or scrambled control (shC) were infected to HN30. Then the cells were treated with indicated drugs for 16 hours and Western blot analysis was performed with the indicated antibodies to determine its effect on cell death.

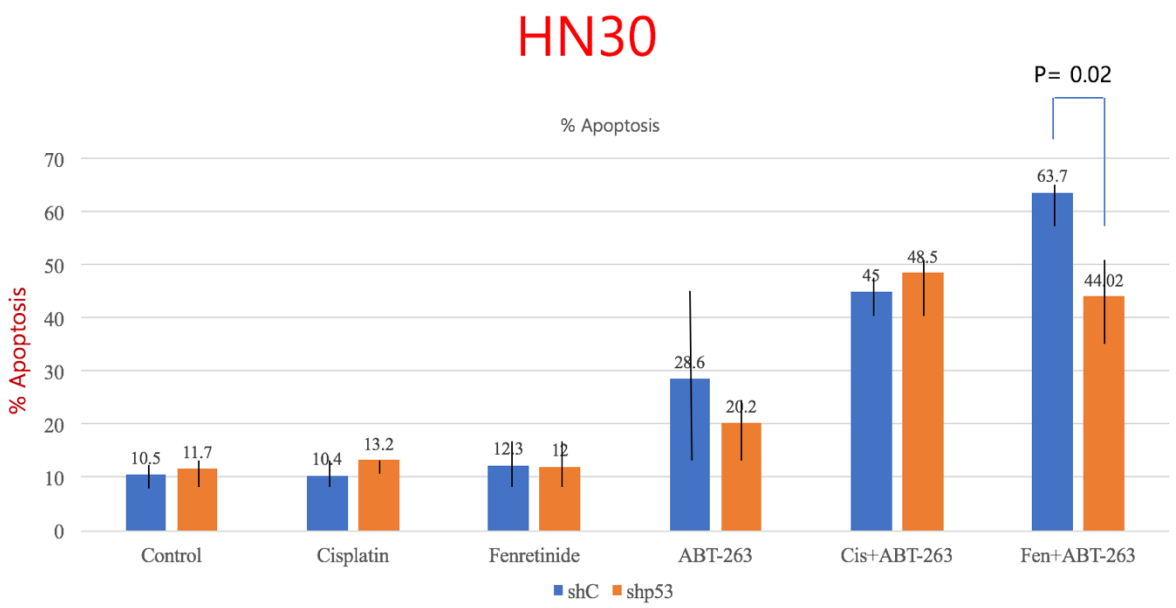


Figure 21: p53 is contributing to fenretinide + ABT-263 combination cell death. The lentiviruses encoding short-hairpin p53 (shp53) or scrambled control (shC) were infected to HN30. Then cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

5.5 Contribution of BIM in combination-induced cell death

BIM is known as BH3-only pro-apoptotic protein that cause the release of cytochrome c from the mitochondria by activating BAX and/or BAK. To examine if BIM is contributing to apoptosis in combination treatments, we introduced short-hairpin RNA (shRNA) for BIM to downregulate its expression. As control, we introduced non-targeting shRNA. Both sh-control and sh-Bim expressing HNSCC cells were treated similarly as the previous experiments. Our result in Figure 22 clearly shows a reduction in the expression level of BIM, indicating that our shBim construct is working well. However, in both cisplatin + ABT-263 and fenretinide + ABT-263 treatments, the expression of cleaved-PARP has not much changed when BIM was knocked down, suggesting that BIM is not contributing to apoptosis.

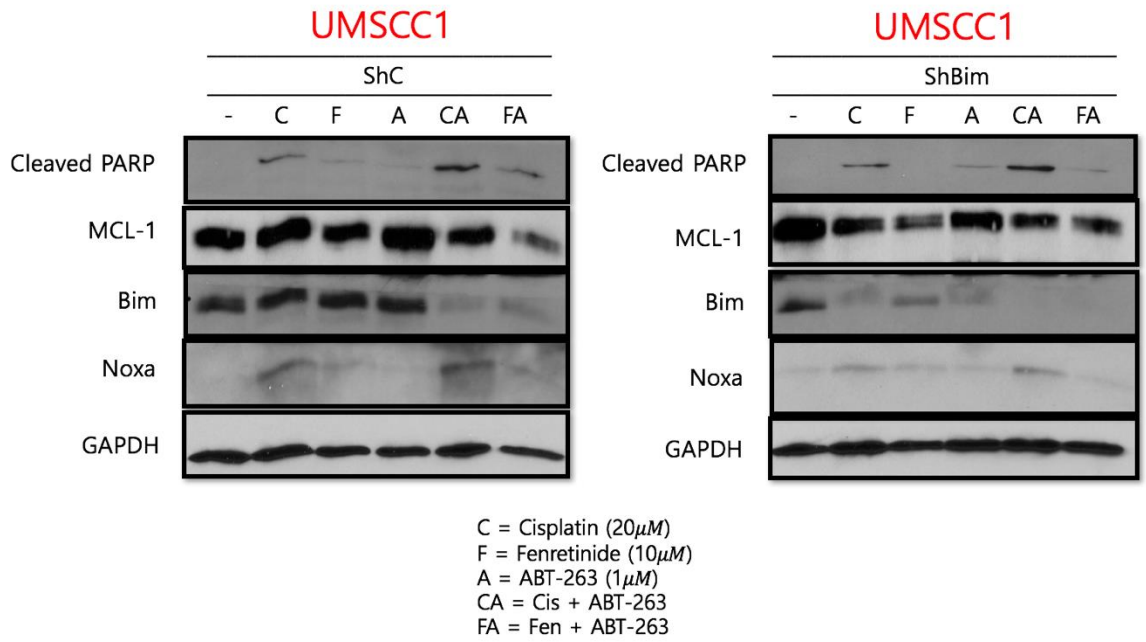


Figure 22: BIM is not involved in combination-induced cell death. The lentiviruses encoding short-hairpin Bim (shBim) or scrambled control (shC) were infected to UMSCC1. Then the cells were treated with indicated drugs for 16 hours and Western blot analysis was performed with the indicated antibodies to determine its effect on cell death.

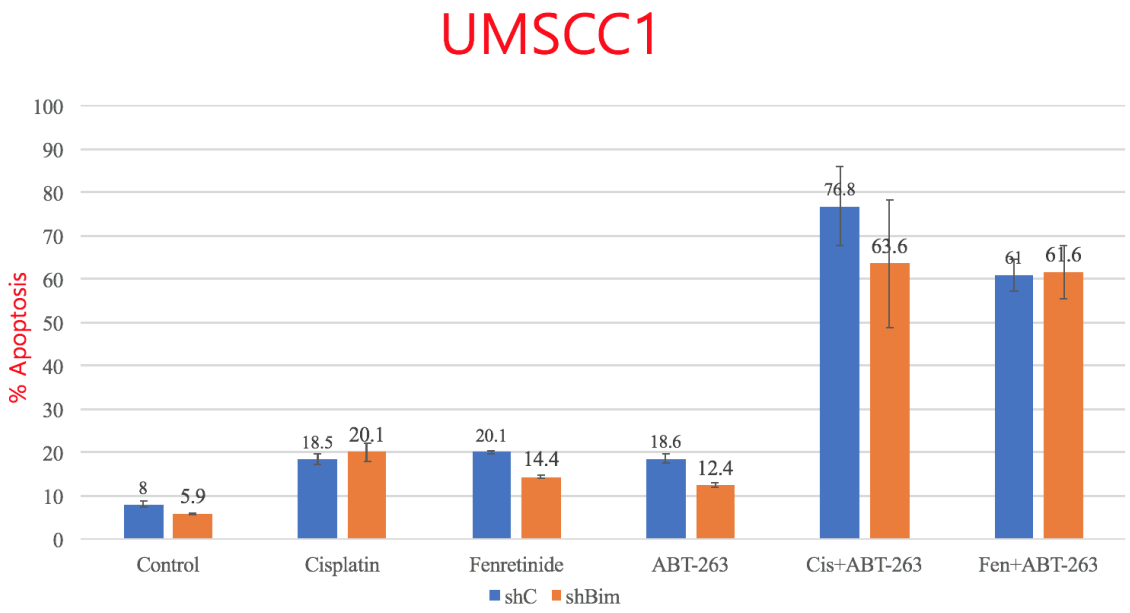


Figure 23: BIM is not contributing to the cell death in combination treatments. The lentiviruses encoding short-hairpin Bim (shBim) or scrambled control (shC) were infected to UMSCC1. Then cells were treated with indicated drugs for 24 hours and fluorescent-activated cell sorting (FACS) analysis was performed to determine the total amount of apoptosis (N = 4). Error bars that represent the standard deviation is depicted by a line through the bar graphs showing the amount of variation of a set of data values.

DISCUSSION

6.1 The sensitivity of cisplatin relatively correlates with the status of p53 in HNSCC cell lines

Treating HNSCC cells with cisplatin results in DNA damage, inducing p53 to prevent DNA replication or induce apoptosis when DNA cannot be repaired. While determining the IC_{50} of the HNSCC cell lines tested, we observed that cell lines with wild-type p53 had higher sensitivity to cisplatin than the cell lines with p53 deletion or non-functional p53 (Table 1 and Figure 11). This results suggest that cisplatin treatment largely induces p53-dependent pathway to induce apoptosis.

6.2 Cleaved-PARP was increased in combination-induced cell death

In HN8 cell line, we observed that cisplatin + ABT-263 combination treatment was not synergistic in inducing apoptosis, whereas fenretinide + ABT-263 treatment resulted in significant increase in cleaved-PARP (Figure 12). Our results from the Western blot analysis further confirmed through the FACS analysis. In cisplatin + ABT-263 treatment, it is known that the activation of p53 induces the expression of Noxa to cause cell death. Whereas in fenretinide + ABT-263, it is known that ER stress activates the transcription factor ATF4 to induce Noxa for apoptosis. Since HN8 does not possess p53 to begin with, it would not be as efficient to induce the pro-apoptotic Noxa to cause cell death by cisplatin. Unlike cisplatin + ABT-263 treatment, fenretinide + ABT-263 treatment was still capable of inducing Noxa to cause significant amount of cell death because induction of Noxa does

not depend on the p53 status (Figure 12). In contrast, both HN30 and UMSCC1 cell lines that harbor wild-type p53, and HN31 that has mutated p53 seemed to have very high sensitivity to the cisplatin. Both cisplatin + ABT-263 and fenretinide + ABT-263 treatments in HN30, HN31, and UMSCC1 have significantly increased the amount of apoptotic cell death with increased expression of Noxa.

6.3 Knock-down of Noxa decreased cleaved-PARP expression in combination treatments

A BH3-only pro-apoptotic protein Noxa is known to sequester anti-apoptotic protein MCL-1 and lead to apoptotic cell death. We observed in HN30 cell line that there was decrease in cleaved-PARP when Noxa was knocked down in both combination treatments (Figure 16). This suggests that combination-induced Noxa expression is required to induce apoptosis. Although both CA (cisplatin + ABT-263) and FA (fenretinide + ABT-263) treatments are largely depending on the expression of Noxa, CA treatment in shNoxa did not result in as much reduction in cleaved-PARP as FA treatment. These results were further confirmed through FACS analysis (Figure 16 and 17). Thus, we investigated whether CA treatment may be inducing other pro-apoptotic proteins, such as Puma. We have observed clear induction of Puma in both cisplatin alone or CA treatment (Figure 16). We speculate that Puma can also be contributing to apoptosis in these treatments and is why down-regulation of Noxa is not resulting in as significant decrease in cleaved-PARP compared to the FA treatment. We have also tested identical treatments in UMSCC1 cell line with shNoxa. We have observed similar results that FA might be depending more on Noxa, whereas CA is partially depending on the expression of Puma to induce apoptosis

(Figure 18).

6.4 Contribution of p53 in combination-induced cell death

p53 is known as a transcription factor that induces the expressions of several pro-apoptotic proteins, such as Noxa and Puma. We wanted to investigate whether p53 is contributing in apoptosis in p53 wild-type HN30 and UMSCC1 cell lines. As previously discussed in ***6.2***, induction of Noxa in FA (fenretinide + ABT-263) treatment would depend on ER stress, whereas induction of Noxa in CA (cisplatin + ABT-263) treatment would depend on activation of p53. We have observed in HN30 that FA treatment was not affected in the amount of cleaved-PARP when p53 was down-regulated. However, CA treatment which is known to depend on p53 seemed to have slight reduction in cleaved-PARP when p53 was knocked down (Figure 20). These results further confirm that CA treatment leads to activation of p53 to induce Noxa to cause apoptosis, whereas FA treatment leads to ER stress to induce Noxa to cause apoptosis.

6.5 Contribution of BIM in combination-induced cell death

BIM is known as BH3-only pro-apoptotic protein that causes the release of cytochrome c to induce apoptosis by activating BAX and/or BAK. Throughout our experiments here, we have seen the expression levels of BIM under combination treatments were substantially decreased in combination treatments. Therefore, we further investigated whether BIM was contributing to cell death with combination treatments by introducing short-hairpin RNA (shRNA) for Bim to downregulate its expression. Figure 22 shows

consistent expressions of cleaved-PARP in both combination treatments even after BIM was down-regulated. While pro-apoptotic protein Noxa is known for its high affinity to pro-survival protein MCL-1, BIM is known to interact with MCL-1, BCL-2, and BCL-X_L. We speculate that BIM may be going through caspase- and/or proteasome-mediated degradation as a consequence of apoptosis. This may be due to strong induction of Noxa in both combination treatments. When Noxa is induced and further bound to MCL-1 to inhibit its pro-survival activity, BIM may be losing its competition against Noxa to interact with MCL-1 and just simply undergoes the degradation process. Results obtained from Western blot analysis were also further confirmed through FACS analysis shown in figure 23.

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VITA

Sung Woo Kim was born on November 19th, 1990, in Bucheon, South Korea. He grew up in South Korea until he immigrated with his family to the United States of America in 2002. Sung received his Bachelor of Science in Biological Sciences from Virginia Tech, Blacksburg, VA in May of 2013. Upon graduating, he completed the Pre-Medical Graduate Health Sciences Certificate program at Virginia Commonwealth University during the spring of 2016. Later that year, he joined the Depart of Physiology & Biophysics and continued his graduate studies upon joining Dr. Hisashi Harada's lab. While pursuing a Master of Science, he was accepted to the dental school in Detroit, MI, and also was selected for the scholarship from the United States Navy as a reserve officer for the dental corps. Sung is currently pursuing his goal of becoming a dentist.