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The Role of Noxa/MCL-1 in Head and Neck Squamous Cell Carcinoma (HNSCC) Treatment

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THE ROLE OF NOXA/MCL-1 IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) TREATMENT

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

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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

PUMA: p53 up-regulated modulator of apoptosis

ERK: Extracellular signal-regulated kinases

JNK: c-Jun N-terminal kinases

GSK3: Glycogen synthase kinase 3

CDK1: Cyclin-dependent kinase 1

CDK2: Cyclin-dependent kinase 2

MULE: MCL-1 ubiquitin ligase E3

PKC: Protein kinase C

HPV: Human papillomavirus

β-TrCP: Beta transducing-containing protein

MDM2: Mouse double minute 2 homolog

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

THE ROLE OF NOXA/MCL-1 IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) TREATMENT

By June Young Lee, B.S.

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Head and neck cancer is the sixth leading type of cancer with 90 percent of head and neck cancer arising from squamous cell lining on the epithelium of the oral and nasal cavity, pharynx, and salivary gland. Even with tremendous achievements on chemotherapeutic drugs and therapies, the long-term prognosis of patients with advanced head and neck squamous cell carcinoma (HNSCC) has shown little improvement over the last three decades. Cisplatin is one of widely used chemotherapeutic drugs for multiple

cancers, including head and neck cancer, but the prolonged use of this drug is limited by its toxicity and by the development of resistance. To overcome these major roadblocks to improved prognosis requires mechanism-based therapeutic strategies to maximize the antitumor effect of drugs while limiting their toxicities. Cisplatin exerts anticancer effects via multiple mechanisms, yet its most prominent mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of BCL-2 family-dependent mitochondrial apoptosis. DNA damage activates a tumor suppressor p53 to induce apoptosis. One of its functions is to induce the expression of several pro-apoptotic proteins such as Noxa, which binds to an anti-apoptotic BCL-2 family protein, MCL-1 (myeloid leukemia cell-1) to inactivate its pro-survival function and induce apoptosis. We examined Noxa expression and apoptosis induced by cisplatin in *p53*-wild-type HN30 and HN31, *p53*-truncated and inactive HN4 and HN12, and *p53*-deleted HN22 and HN8 HNSCC cell lines. We found that Noxa was induced in HN30 and HN31 cells and down-regulation of Noxa by shRNA (short-hairpin RNA) decreased apoptosis, indicating Noxa contribution to cisplatin-induced apoptosis. Interestingly, cisplatin treatment induced Noxa and apoptosis even in *p53*-deleted HN22 and HN8 cells, suggesting the existence of the p53-independent pathways for the induction of Noxa. Based on these observations, we hypothesized that modulation of Noxa/MCL-1 axis could mimic cisplatin-induced cell death. We found that Noxa overexpression induced cell death in all cell lines tested regardless of p53 status. This finding could be applicable as a potential therapeutic strategy to treat head and neck cancer.

INTRODUCTION

1.1 Cancer

Cancer is a genetic disease caused by altering genes in our body that control the growth and dividing functions. This leading cause of death worldwide is estimated to kill about 589,430 Americans in 2015 and about 1.65 million new cases are expected in 2015 (www.cancer.gov). According to WHO, new cases of cancer will increase from 14 million in 2012 to 22 million within the next two decades. Among the various cancer types, the most common causes of death are lung, liver, stomach, colorectal, breast, and esophageal cancer (www.who.org). With increasing number of deaths and new cases, it is important for us to comprehend the molecular mechanisms of tumorigenesis and cell death to specifically enhance it in cancer cells.

There are many known factors that increase the risk of cancer. Cigarette smoking is highly related to increase the risk for many types of cancer such as bladder, kidney, esophageal, lung, oral cavity, and stomach cancers. It is believed that cigarette smoking causes about 30% of all cancer deaths in United States (www.cancer.gov). Infections by viruses and bacteria can also increase the risk of cancer. For example, human papillomavirus (HPV), Hepatitis B and C viruses, and helicobacter pylori can increase the risk for cancers. Radiation is another factor to increase in cancer development. Ultraviolet radiation from sunlight, x-rays, CT scans, and fluoroscopy are some of the examples for

radiation. Factors like diet, alcohol consumption, physical activity, obesity, and environmental risk factors may affect the risk of cancer.

1.2 Head and Neck Cancer

Head and neck cancer is a general term that branches out to a specific type and place of cancer, and 90 percent of head and neck cancers arise from squamous cell lining the epithelium of the oral and nasal cavity, pharynx, larynx, paranasal sinuses, and salivary glands [3]. Head and neck squamous cell carcinoma (HNSCC) is the sixth leading type of cancer with estimated 650,000 of new cancer cases and 350,000 of cancer deaths worldwide per year [2]. About 3 percent of all cancers in the United States are believed to be head and neck cancer [12], which occurs twice as common among men as it does among women [1]. It is known that 80% of cases eventually become metastatic to invade the regional lymph nodes of the neck [2] and five-year survival rate of patients with HNSCC is 40 to 50 percent.

The two highest risk factors for head and neck cancer are alcohol and tobacco (chewing and smokeless) use. About 75 percent of head and neck cancer are caused by tobacco and alcohol use and people have higher risk when they use both compared to people who use one or the other [8-10]. These high risk factors affect oral cavity, oropharynx, hypopharynx, and larynx cancers [4-7]. Another high risk factor that is currently increasing the cause of oropharyngeal cancer in tonsils and base of tongue is with infection of human papillomavirus-16 (HPV-16) [11]. Currently in the United States, more than half of oropharyngeal cancer is linked to HPV-16 [16].

Treatment for head and neck cancer varies with where the cancer is located and the stage of the cancer. Majority of HNSCC treatment begins with radiation or chemotherapy, and followed by surgery, however, treatment options are plan accordingly to each patient. With head and neck cancer, there are five different stages starting from zero to four. At stage 0, the tumor just begins to grow and is not found in deeper tissues. A primary tumor with 2 cm or smaller in size with no cancer cells in nearby structures or lymph nodes is considered as stage I. Tumor size of 2 to 4 cm is stage II and tumor size greater then 4 cm is considered as stage III. Stage IV in head and neck cancer has three different types (A, B, and C). Stage IVA means that the tumor is in any size but is not found in lymph nodes or other parts of the body. Stage IVB represents that the tumor has migrated to lymph nodes and Stage IVC means that the tumor has spread to lymph nodes as well as other distant sites of the body (www.cancercenter.com).

For early or localized disease (stage I-II), either surgery or radiation therapy is needed and patients with locally advanced disease (stage III-IV), both chemotherapy and radiation therapy are required to increase the cure rate. Many different types of chemotherapeutic drugs are used to cure head and neck cancers. The drugs such as cisplatin, paclitaxel, and carboplatin are widely used in conjunction to treat the tumor.

1.3 Cisplatin

Cisplatin is a chemotherapeutic drug known as “the penicillin of cancer”, because of its wide use of treating cancers including testicular, head and neck, bladder, esophageal, non-small cell lung, stomach, breast, cervical, and prostate. Michel Peyrone

created cisplatin in mid-19th century and it wasn't used until 1960s for biological effects. Cisplatin was first researched with *E. coli.* and scientists have found that it inhibits reproduction and prevent binary fission [15]. It was then researched on rats and the Food and Drug Administration approved cisplatin in 1978 for multiple cancer treatments.

Cisplatin (cis-PtCl2(NH_3)$_2$) consists of 11 atoms with platinum in the center (Figure 1). Even though cisplatin is widely used as a chemotherapeutic drug to cure cancer, we do not know the specific mechanisms on how cisplatin induces particular proteins to stimulate cancer cell death. The platinum in cisplatin is the key to induce cell death in cancer cells. Cisplatin works as a simple nucleophilic substitution reaction. When cisplatin is intravenously injected into the body, the chlorine atom gets replaced by a hydroxyl group and binds covalently to the DNA in the cell (Figure 2) [15]. Cisplatin crosslinks with DNA as monoadduct, interstrand crosslink, intrastrand crosslink or DNA-protein crosslink which acts on adjacent N-7 guanine forming 1, 2 intrastrand crosslink [13, 14]. Also, cisplatin can bind to a protein on one side and bind to DNA on the other side. This cisplatin-protein interaction plays a critical role to protect cisplatin from the DNA repair mechanisms in the cell [15]. Failure to repair the DNA causes replication arrest and cell death occurs.

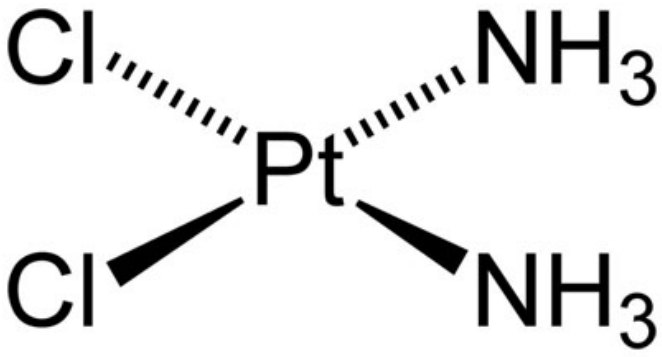


Figure 1: Structural formula of cisplatin. The chemical name for cisplatin is *Cis*-Platinum(II)diamine dichloride (cis-$PtCl2(NH_3)_2$)

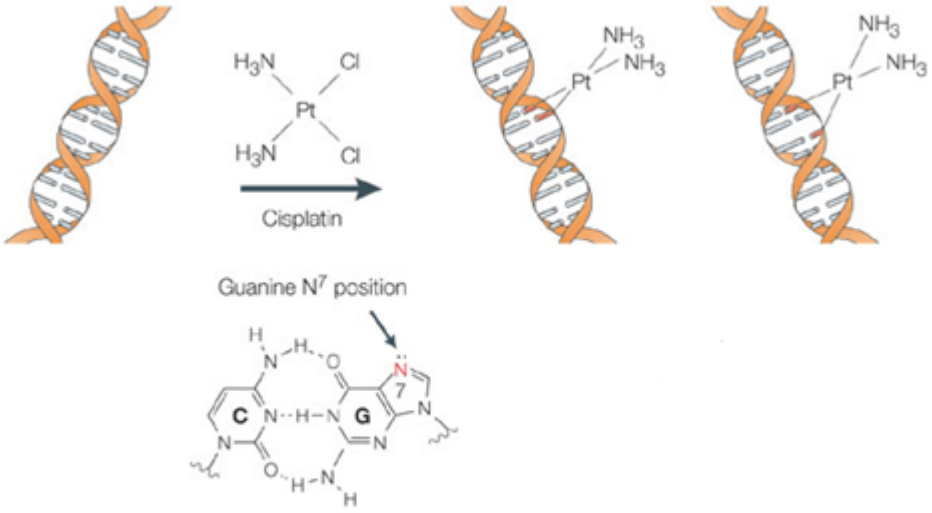


Figure 2: Cisplatin crosslinks with DNA and inhibit replication to induce apoptosis [modified26]

1.4 Cell Death

Apoptosis, autophagy, and necrosis are three major types of cell death. Apoptosis (from Greek *apo*, "by, from" and *ptosis*, "fall")-stimulated cells shrink due to cytoskeleton collapses, nuclear envelop disassembly and fragmentation as well as cleavage of DNA. Over all, cell death through apoptosis is neat and clean as the bodies of blebs are well cleared out by phagocytic cells. Autophagy (from Greek *auto*-, "self" and *phagein*, "to eat") is another type of cell death that is not as well studied as apoptosis. Interestingly, autophagy can increase cell survival when the cell suffers from starvation by breaking down its own cellular components to maintain cellular energy level. Thus, autophagy is considered as an adaptive response to stress that can promote survival or death [17]. Necrosis is stimulated by extreme stress towards the cell or tissue by infection, toxins, or trauma, and the cell swells and bursts as the integrity of the cell membrane decreases. Unlike apoptosis, necrosis is always very harmful and can be fatal.

Apoptosis, also known as programmed cell death, manages proper homeostasis of highly controlled multicellular organism. If apoptosis does not occur properly, cells could develop into cancers and autoimmunity. On the other hand, immoderate cell death causes cardiovascular disease like stroke and heart attack and neurodegenerative disorders like Alzheimer, Parkinson, and Huntington disease. With the importance of homeostasis, regulation of apoptosis must be in absolute control in order for proper function of the multicellular organisms.

Two main pathways of apoptosis are extrinsic (cytoplasmic) and intrinsic (mitochondrial) pathways (Figure 3). In the extrinsic pathway, ligands such as Fas-L or

TNF (tumor necrosis factor) bind to its corresponding death receptors FAS or TNFR1 (tumor necrosis factor receptor 1), respectively, and the intracellular regions of the receptors engage specific adapter proteins to cleave pro-caspases to be activated. The activated initiator caspases (e.g. caspase-8 or -10) have ability to activate other pro-caspases (e.g. caspase-3), resulting in amplifying proteolytic cascade. For example, Fas-FasL complex recruits FADD (Fas-associated death domain) protein and pro-caspase-8 is cleaved to become an active caspase-8. Activated caspase-8 activates caspase-3, which triggers apoptosis [18]. When cells are disrupted by stress, damaged cytoskeleton and DNA can activate the intrinsic pathway to initiate apoptosis. This pathway engages the BCL-2 family proteins that regulate cell death through either induction or inhibition of pro- and anti-apoptotic proteins. Activation of pro-apoptotic proteins creates channels at the outer mitochondrial membrane, resulting in the release of cytochrome c from the mitochondrial intermembrane space to cytosol through MOMP (mitochondrial outer membrane permeabilization). Then cytochrome c binds to APAF1 (apoptotic protease activating factor 1) to be activated. Activated APAF1 binds to an initiator caspase, caspase-9 to form a complex called apoptosome, which activates an executor caspase, caspase-3 to initiate apoptosis [19].

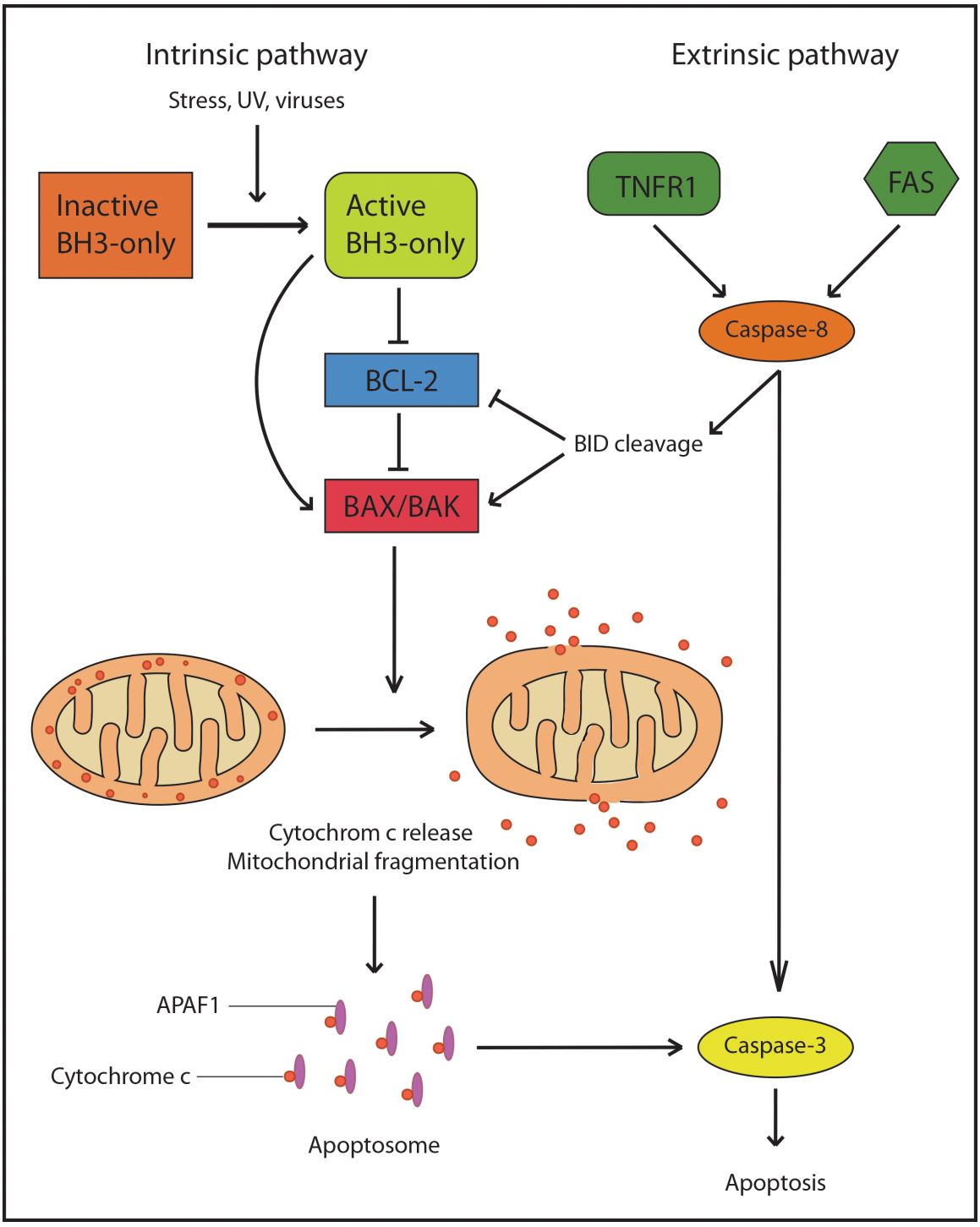


Figure 3: Simplified diagram of the intrinsic and extrinsic pathways of apoptosis [Modified 27].

1.5 BCL-2 Family

A group of proteins that regulate mitochondrial pathway of apoptosis is called the BCL-2 family of proteins. These proteins only share a number of specific sequences homology called BH (BCL-2 homology) domains. The family is subdivided in three classifications according to its function and by which BH domain it has. Currently, four groups of BH domains are identified (BH1, BH2, BH3, and BH4) and the first group is classified as pro-apoptotic multi-domain BCL-2 effectors. The proteins such as BAK and BAX contain multiple BH domains that stimulate apoptosis by triggering MOMP. BCL-2, BCL-X_L, MCL-1, A1, and BCL-w are known as the second classification, which refers to the anti-apoptotic BCL-2 proteins. These proteins inhibit the function of pro-apoptotic proteins by the protein-protein interaction and prevent MOMP. The third group that consists of BAD, BID, BIM, PUMA, Noxa, etc. is called as BH3-only proteins. These proteins only share homology with the BH3 domain and they have the ability to bind and inhibit anti-apoptotic BCL-2 proteins. Some BH3-only proteins such as BIM and BID also have the ability to directly activate BAX and BAK proteins to promote apoptosis. Overall, BH3 only proteins promote MOMP and apoptosis.

BAK (BCL-2 homologous antagonist killer) and BAX (BCL-2 associated X protein) have unique mechanisms to induce apoptosis. Without apoptotic stimuli, BAX is normally soluble in cytosol and BAK is inactivated by the interaction with MCL-1 or BCL-X_L at the outer membrane of the mitochondria. The BH groove of the anti-apoptotic BCL-2 proteins prevents MOMP and apoptosis by binding to the BH3 domain of BAK or BAX to interfere the oligomerization. The BH grooves exist in all of the anti-apoptotic

BCL-2 proteins, but they need to be present in a hydrophobic environment such as the outer mitochondrial membrane. As apoptosis is induced, BAX is translocated to the mitochondria. On the other hand, BAK is freed from BAK-MCL-1 or BAK-BCL-X_L complex. Conformational changes of BAK or BAX expose the BH3 domain to open their grooves, bind to a BH3 domain of either BAX or BAK, and form a dimer. Then BAX and/or BAK oligomerize and integrate into the outer mitochondrial membrane to form channels, which allow cytochrome c to release from the mitochondria followed by caspase activation and apoptosis.

There are total of 11 BH3-only proteins identified in human and they have abilities to bind to the BH grooves of the anti-apoptotic BCL-2 proteins, but there is specificity for the interaction between BH3-only proteins and anti-apoptotic members. For example, BIM and BID are capable of interacting with all anti-apoptotic proteins. Noxa has a strong affinity to interact with MCL-1, however it has much less capability to bind to other anti-apoptotic proteins such as BCL-X_L and BCL-2. In contrast, BAD and BMF have affinities only to BCL-X_L and BCL-2, but not MCL-1. BID and BIM have another mechanism of binding to not only the anti-apoptotic proteins, but also BAX and BAK to trigger the oligomerization, which causes MOMP and initiates apoptosis.

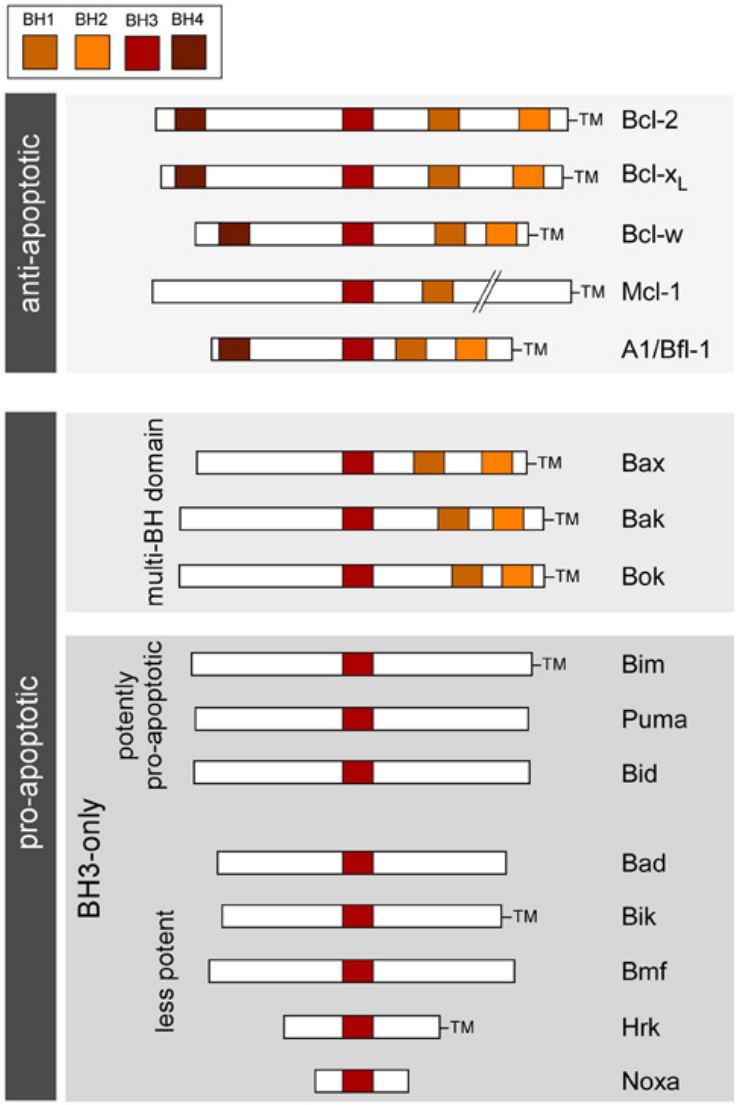


Figure 4: List of the BCL-2 family of proteins. All BCL-2 family of proteins contains short BH (BCL-2 homology) domains [32].

1.6 MCL-1 and Noxa

MCL-1 (myeloid cell leukemia 1) was originally found in 1993 in the differentiating myeloid leukemia cell line [20]. It is composed of 350 amino acids in human and plays a critical role in regulating cell death and survival in both normal and cancer cells. The effect of MCL-1 is to prevent cell death by sequestering pro-apoptotic family members such as BAK or the BH3-only proteins (Noxa, BID, and BIM). Studies have shown that deletion of *Mcl-1* in mice causes embryonic lethal phenotype, representing the significance of MCL-1 in early development [21]. The activity of MCL-1 is regulated by its high turnover rate, which is mediated by phosphorylation, ubiquitination, and degradation. A region called PEST in MCL-1 governs MCL-1's stability [35]. The PEST domain of MCL-1 contains multiple phosphorylation sites for protein kinases such as ERK, JNK, GSK3, CDK1 and CDK2. For example, GSK3 (Glycogen synthase kinase 3) is a kinase that is activated by decrease in activation of AKT. When GSK3 is activated, MCL-1 is phosphorylated and ultimately is degraded. However, ERK-mediated phosphorylation of MCL-1 stabilizes the MCL-1 protein. It has been demonstrated that MCL-1 ubiquitin ligase E3 (MULE) and beta transducing-containing protein (β-TrCP) are both E3-ubiquitin ligases that regulate MCL-1 stability. Other mechanisms that control the levels of MCL-1 are caspases and Granzyme B that cleave distinct sites within the N-terminus of MCL-1 during the progression of apoptosis. Cleaved MCL-1 reduces its ability to halt apoptosis stimulation.

Noxa is a pro-apoptotic BH3-only molecule that was originally identified as p53 target gene [24]. The tumor suppressor p53 induces apoptosis in cells that are jeopardized

by cellular stresses. It has been shown that decreasing endogenous Noxa expression levels suppresses p53-dependent apoptosis. Noxa is known to affect the function of MCL-1 by binding to its BH3 domain to BH3 domain of MCL-1. Noxa specifically binds to and recruits MCL-1 from cytosol to the mitochondria [34]. Translocation of MCL-1 initiates its phosphorylation and subsequent ubiquitination, which triggers proteasome-mediated degradation [22]. Different types of signals can induce Noxa expression and its transcription and protein expression can be activated by both p53-dependent and -independent manner. For example, DNA damage by cisplatin triggers p53 to induce Noxa, whereas a mitogenic stimulation activates PKC (protein kinase C) and induces Noxa independent of p53 [23]. DNA damage by ultraviolet radiation is also known to stimulate Noxa in fibroblasts and skin keratinocytes, and the significance of Noxa has been demonstrated in Noxa knockout mice [31].

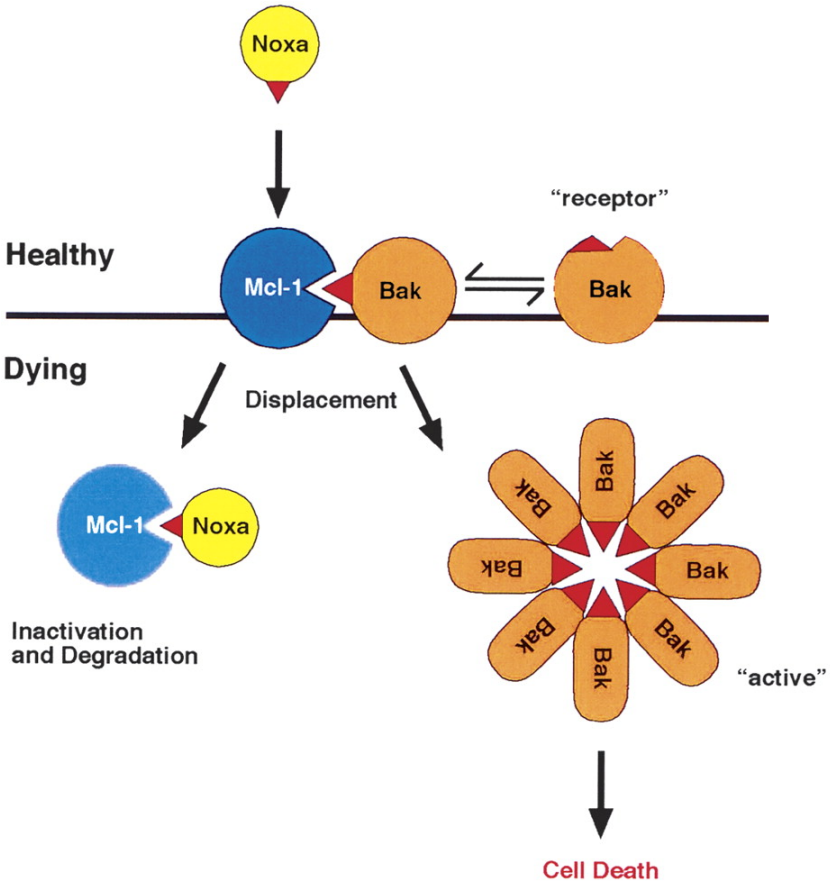


Figure 5: Noxa sequesters MCL-1 to release BAK form the BAK-MCL-1 complex and BAK oligomerizes to induce cell death [Modified 29].

1.7 p53

A tumor suppressor, p53 has many important functions of anti-cancer functions. This 53 kDa protein in human has the abilities to prevent abnormal cell proliferation that might turn into tumors. p53 can also be activated by many stresses such as ultraviolet, x-rays, chemotherapeutic drugs, and DNA synthesis inhibitors. All of these stresses rapidly increase the level of p53 protein by phosphorylating p53 by kinases such as Chk1, Chk2, and ATM, which prevents p53 degradation [33]. High concentration of p53 stops cell growth by holding the cell cycle at G1/S (cell cycle arrest) and induces DNA repair proteins to fix the damage and to prevent replication of the damaged DNA. If it is irreparable, p53 degradation is continuously blocked by phosphorylation. This initiates p53 to act as a transcription factor to induce the expressions of several pro-apoptotic target genes such as Noxa, Puma, and BAX for the apoptosis induction [33]. If the DNA is repaired, signals that protect p53 degradation disappear and the cell cycle resumes as the cell enters into the S phase to replicate its DNA. When the cell is in a normal state, p53 is rapidly degraded by a protein called Mdm2 (in mouse cells) and MDM2 (in human cells) [33]. This protein binds to the N-terminus of p53 when the kinases (Chk1, Chk2, and ATM) are not stimulated. The p53-Mdm2 complex triggers polyubiquitylation and proteasome-mediated destruction.

In order for tumors to thrive, tumor related mutant *p53* alleles often carry point mutations in the reading frames that create missense codons (amino acid substitutions). Mutated p53 produces either a non-functional or a gain-of-function protein, which allows the tumor to grow uncontrollably.

HYPOTHESIS

Cisplatin exerts anticancer effects via multiple mechanisms, yet its most prominent mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of BCL-2 family-dependent mitochondrial apoptosis. DNA damage activates a tumor suppressor p53 to induce apoptosis. One of its functions is to induce the expression of several pro-apoptotic proteins such as Noxa, which binds to an anti-apoptotic BCL-2 family protein, MCL-1 to inactivate its pro-survival function and induce apoptosis. We hypothesized that **Noxa-MCL-1 axis is a crucial determinant of the efficacy of DNA-damaging agents for HNSCC treatment.**

SPECIFIC AIMS

3.1 Determine how Noxa and MCL-1 regulate apoptosis induced by cisplatin in HNSCC cells.

Toxicity levels of cisplatin to a panel of HNSCC cell lines will be measured. Lentivirus-mediated shRNA (knock-down of Noxa) will be introduced to examine the contribution of Noxa to cisplatin-induced cell death.

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

The cells will be also infected with lentivirus harboring Flag-tagged Noxa cDNA to overexpress Noxa and cell death will be determined. Immunoprecipitation will be performed with anti-Flag antibodies to examine the binding affinity between Noxa and MCL-1.

MATERIALS AND METHODS

4.1 Cell lines and Cell Culture

HN4, HN12, HN22, HN8, HN30, and HN31 head and neck squamous cell carcinoma (HNSCC) cells were provided by Dr. Andrew Yeudall (Virginia Commonwealth University, VA). 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 and HN31/sh-Noxa cells were maintained with 2 μg/mL of puromycin (InvivoGen, San Diego, CA) for selection. HN30/pCDH-Noxa and HN31/pCDH-Noxa cells were maintained using G418 (Fisher Scientific, Waltham, MA) at 0.4 mg/mL concentration 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). Flag-tagged Noxa cDNA was cloned into pCDH-EF1-MCS-IRES-neo (System Biosciences, Mountain View, CA). The constructs were transfected into 293T packaging cells along

with the packaging plasmids (Addgene; Cambridge, MA) and the lentivirus-containing supernatants were used to HNSCC cell lines.

4.3 Chemicals and Antibodies

Cisplatin (sc-200896) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for BIM, BCL-X_L (54H6), Cleaved PARP (Asp214), 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); Alpha-Tubulin (sc-8035), BAX (sc-493), and p53 (sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA); BAK from Millipore (Darmstadt, Germany); Noxa (114C307.1) from Thermo Fisher Scientific (Waltham, MA). ECL 2 Western blotting substrate (80196) was purchased from Thermo Scientific (Rockford, IL). Cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Mannheim, Germany).

4.4 Western Blot and Immunoprecipitation Analysis

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)][30]. Protein concentrations from the lysates were measured by spectrophotometric analysis with Bradford method (Bio-rad, Hercules, CA), and equal

amounts of samples were loaded into SDS-polyacrylamide gel. The gel was electrophoresed at 200 volts for approximately 40 minutes and the proteins were transferred onto a nitrocellulose membrane (Fischer Scientific, Pittsburgh, PA) with 100 volt for one hour. The nitrocellulose membrane was then blocked with 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).

To perform immunoprecipitation (IP), 500 µg of protein sample was prepared to match a total volume to be 500 µL. The samples were then incubated with 40 µL of Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) for two hours on a rotating table at 4°C. After the incubation, the beads were washed by centrifugation for 30 seconds at 6000 rpm. Supernatants were removed and 1 mL of CHAPS buffer was added and incubated again on the rotating table for 5 minutes at 4°C. This step was repeated three times. The beads were then re-suspended in 30 µL CHAPS buffer with 5x sample buffer. The samples were loaded to SDS-PAGE gels.

4.5 Cell Toxicity (WST-1) Assay

Head and neck squamous cell carcinoma cells were seeded in triplicate in microtiter plates (96 wells) with concentration of $1x10^4$ cells per well in 150 µL medium. The following day, cells were treated with different concentration of cisplatin and 48 hours later, 4 µL of WST-1 reagent were added to the cells. 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).

4.6 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}.

4.7 Quantitative RT-PCR

To check if cisplatin treatment is effecting the mRNA levels of Noxa and MCL-1 in HN22 and HN8 cells, total RNA was extracted with the RNA Mini prep kit (Genesee Scientific, San Diego, CA). One-microgram of RNA was reverse-transcribed by High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Taqman Gene Expression Assay probe/primer (Applied Biosystems) of MCL-1 (Hs03043899) and Noxa (Hs00560402) were used to amplify the cDNAs with fluorescence thermocycler (Applied Biosystems; 7500HT Fast Real-time PCR system) and were analyzed based on the expression level of GAPDH (Applied Biosystems, 4352934E) with SDS2.2 software.

RESULTS

5.1 HNSCC cell lines

Following list of head and neck squamous cell carcinoma cell lines were used in this study and their primary origin and main characteristics are outlined in Table 1.

Table 1. HNSCC Cell lines

Patient	Cell line	Origin	p53 status
A	**HN4**	Base of tongue	Truncated
	HN12	Lymph node	Truncated
B	**HN22**	Epiglottis	Deleted
	HN8	Lymph node	Deleted
C	**HN30**	Pharynx	Wild-type
	HN31	Lymph node	Wild-type

All three cell lines from the lymph node are metastatic. HN4 and HN12 cell lines have a mutation at the splicing donor site of exon 7 [28], thus produce a truncated form of p53, which is non-functional. The *p53* genes are deleted in HN22 and HN8 cells, whereas HN30 and HN31 cells possess wild-type *p53* gene [28]

5.2 The expression of the BCL-2 family proteins and p53 in HNSCC cell lines

We analyzed the endogenous expression levels of various BCL-2 family proteins and p53 (Figure 1a and 1b). Protein expression levels were determined by immunoblot analyses and tubulin or GAPDH (housekeeping proteins in cells) were employed as a control to demonstrate equal loading.

The expression levels of BAK, BAX, and MCL-1 were relatively similar. BIM expression level in HN30 was very low compared to other cell lines, whereas HN31 had high Noxa expression level. The p53 levels in all cell lines correspond to the gene status of *p53*. In Figure 6b we observed a slight protein expression of ~40 kDa in HN4 and HN12 cell lines, no expression in HN22 and HN8 cell lines, and high expression of p53 in HN30 and HN31. The faint bands represent the truncated, non-functional p53 in HN4 and HN12 cells. The p53 gene was deleted in HN22 and HN8 cells, thus there was no expression. Wild-type p53 was expressed in HN30 and HN31, however, HN31 expressed significantly higher amount of p53 compared to HN30.

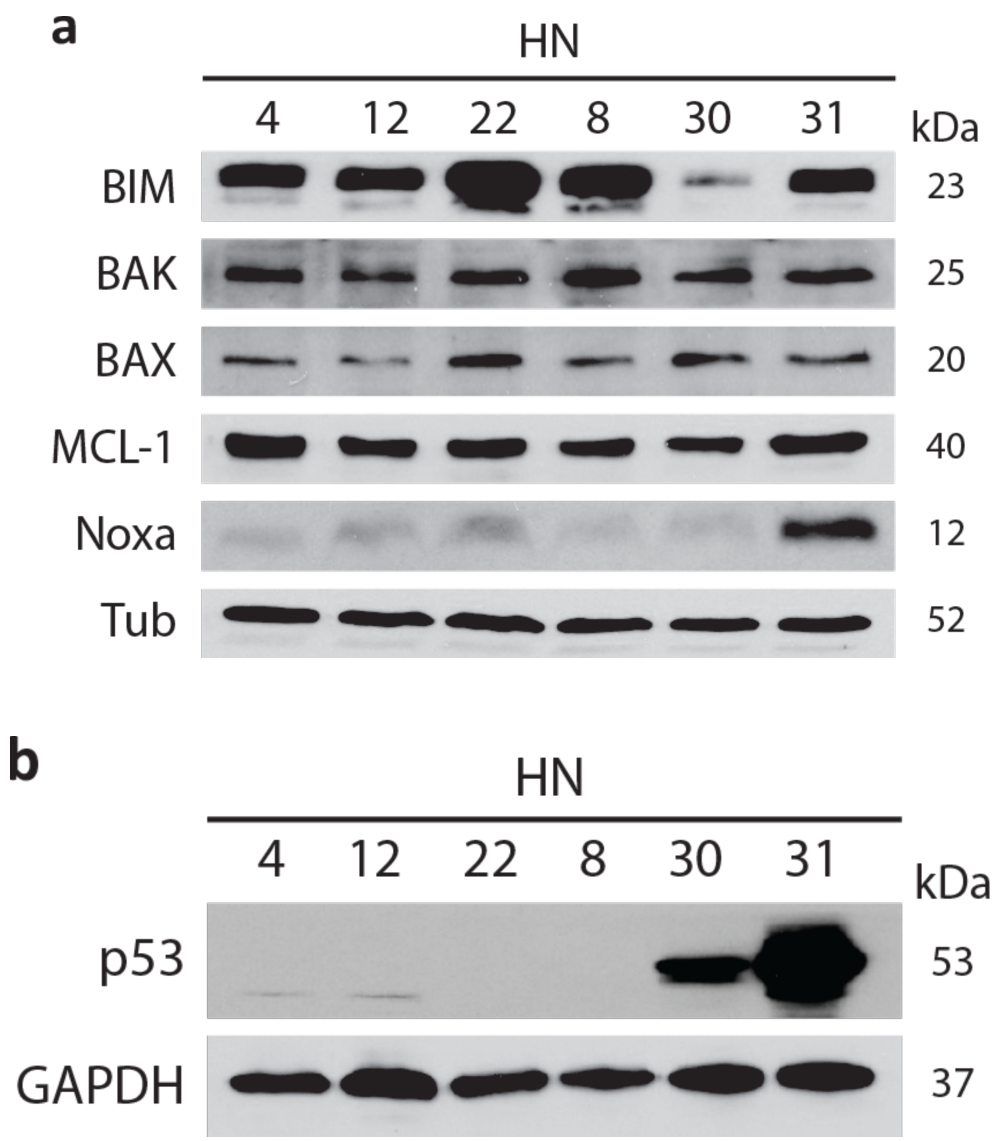


Figure 6: Expression levels of the BCL-2 family of proteins and p53. (**a**) Equal amounts of total lysates of HNSCC cells were subjected to immunoblot analyses using the indicated antibodies. (**b**) Total cell lysates were subjected to immunoblot analysis using anti-p53 antibodies. HN30 and HN31: wild-type p53; HN4 and HN12: truncated/non-functional p53; HN22 and HN8: p53 was deleted.

5.3 Cisplatin sensitivity in HNSCC cell lines, IC_{50}

IC_{50} of all cell lines with cisplatin treatment was determined with WST-1 assay. When the cells are metabolically active, WST-1 (tetrazolium) salt is turned into formazan dye, which can be quantified by a spectrophotometer at 450 nm (Figure 7).

We found that IC_{50} values relatively correlated with p53 status in the cell lines. p53-truncated HN4 and HN12 had the highest IC_{50} of 75 μM and 63 μM, respectively and *p53*-deleted HN22 and HN8 cell lines both had IC_{50} of 50 μM . The p53-wild-type cell lines, HN30 and HN31 were the most sensitive cells to cisplatin. Twenty μM of cisplatin was required to kill half of HN30 and HN31 cell lines (Table 2 and Figure 8).

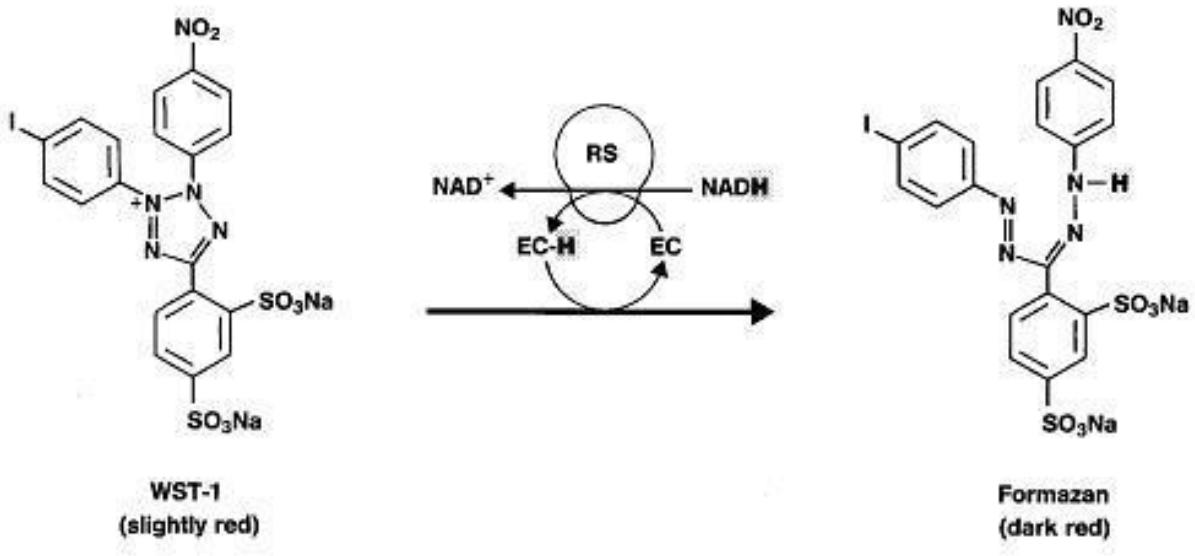
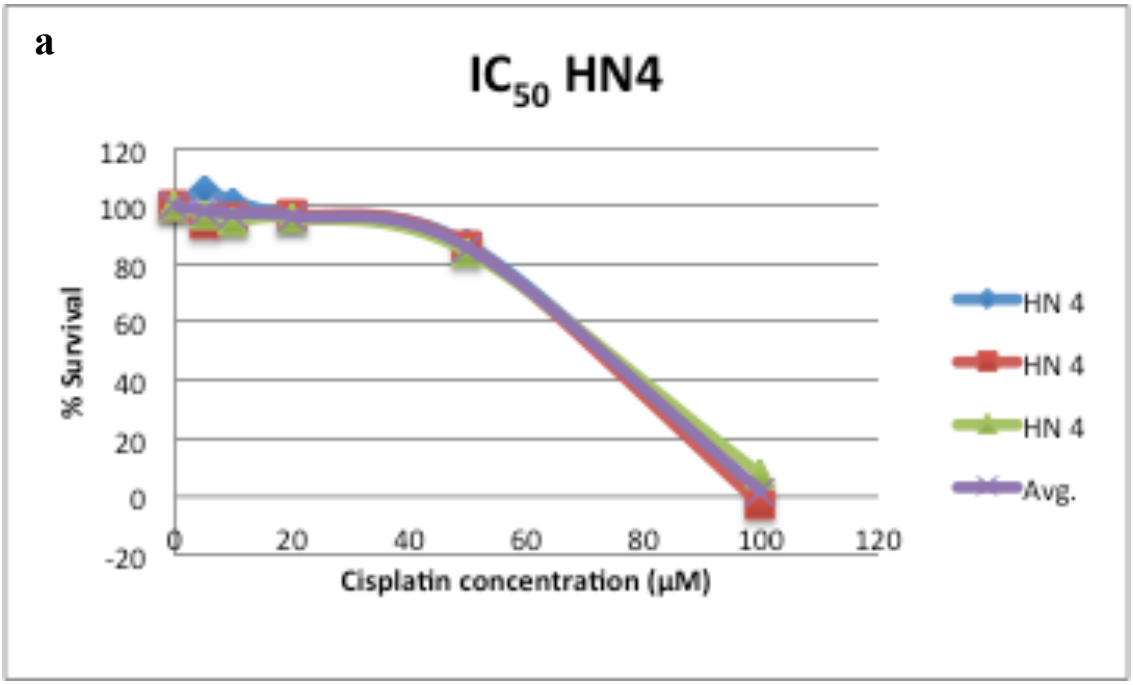
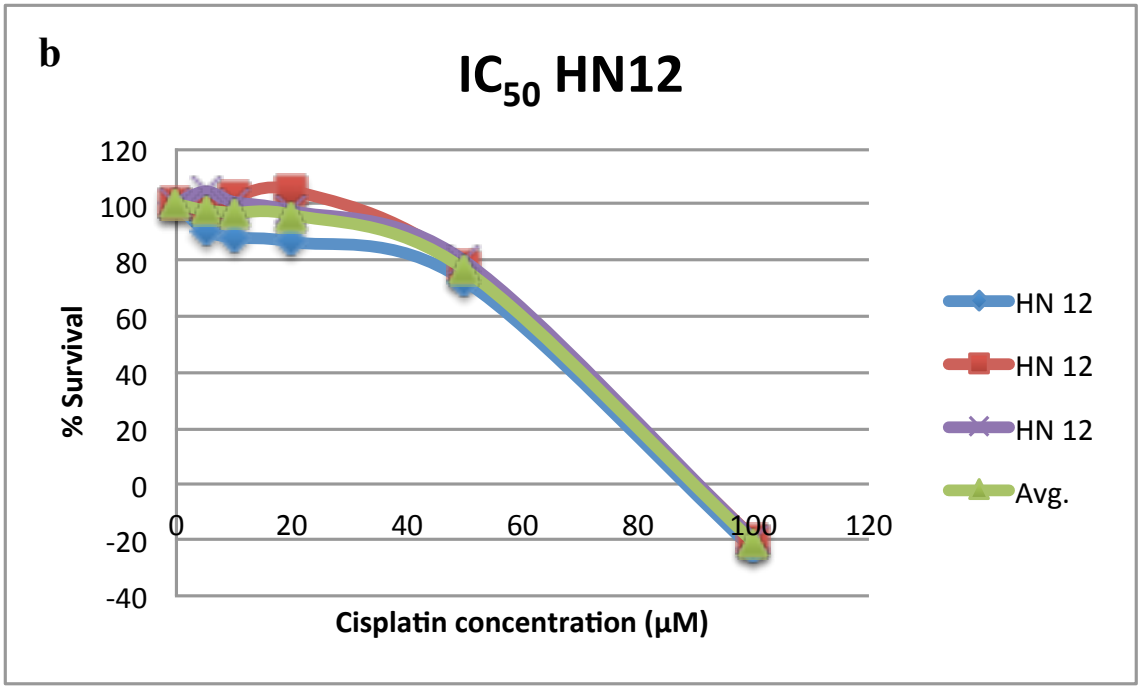
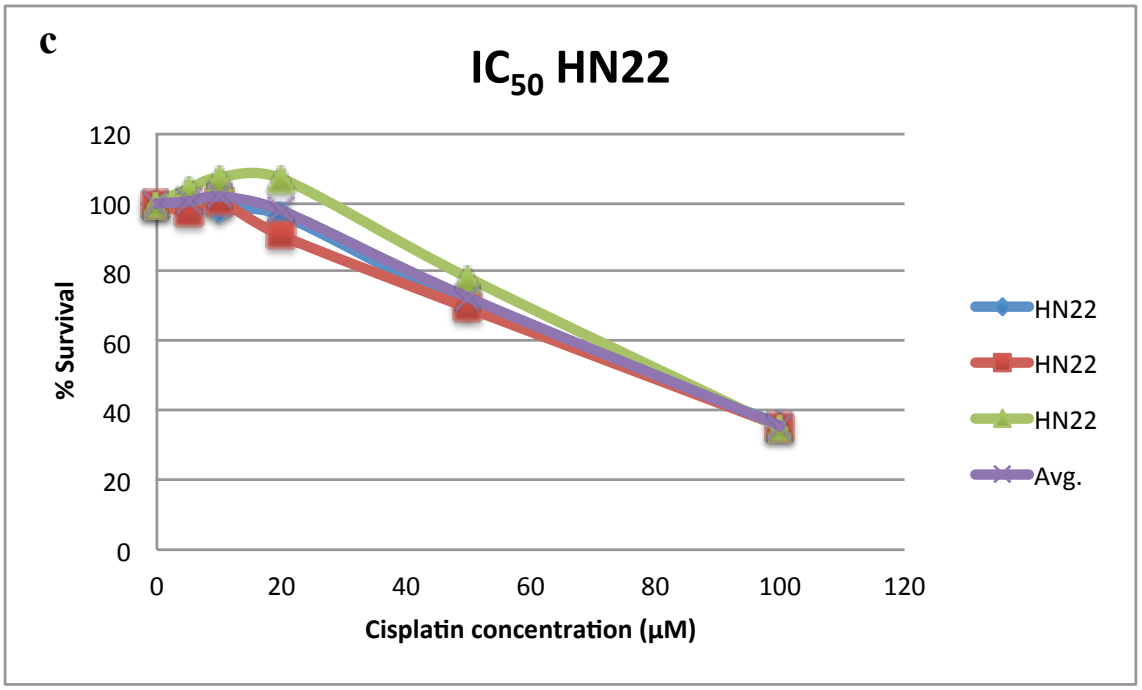


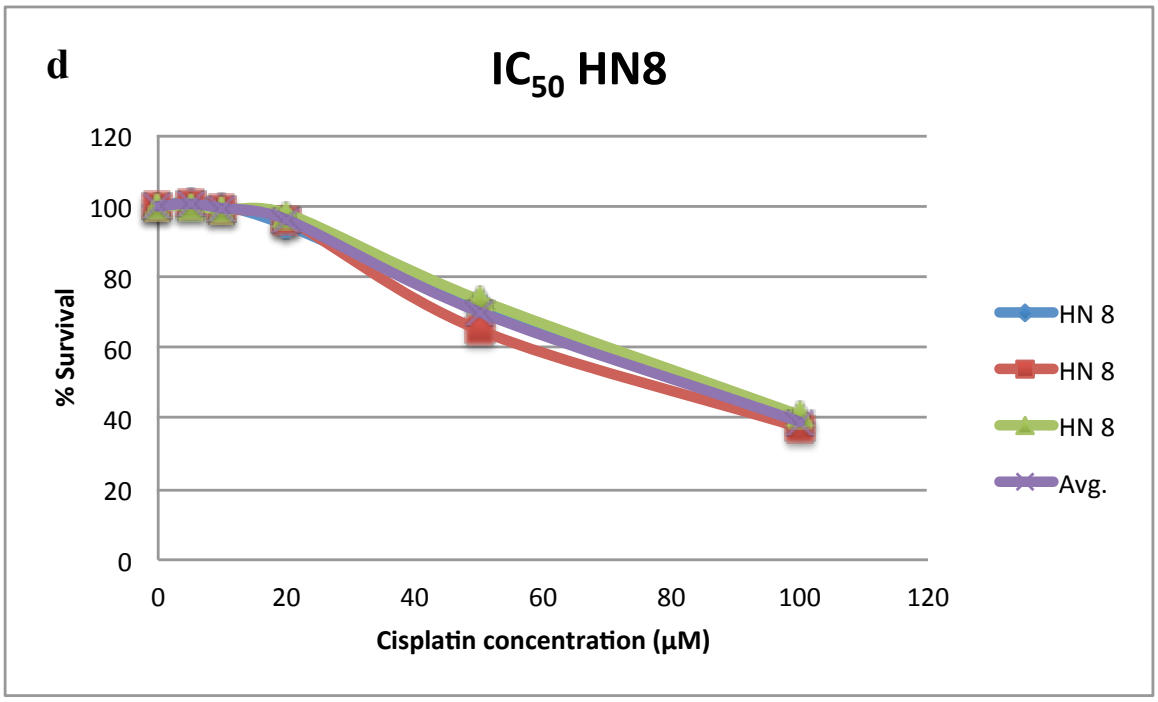
Figure 7: Cleavage of the tetrazolium salt WST-1 to formazan. (EC = electron coupling reagent, RS = mitochondrial succinate-tetrazolium-reductase system)

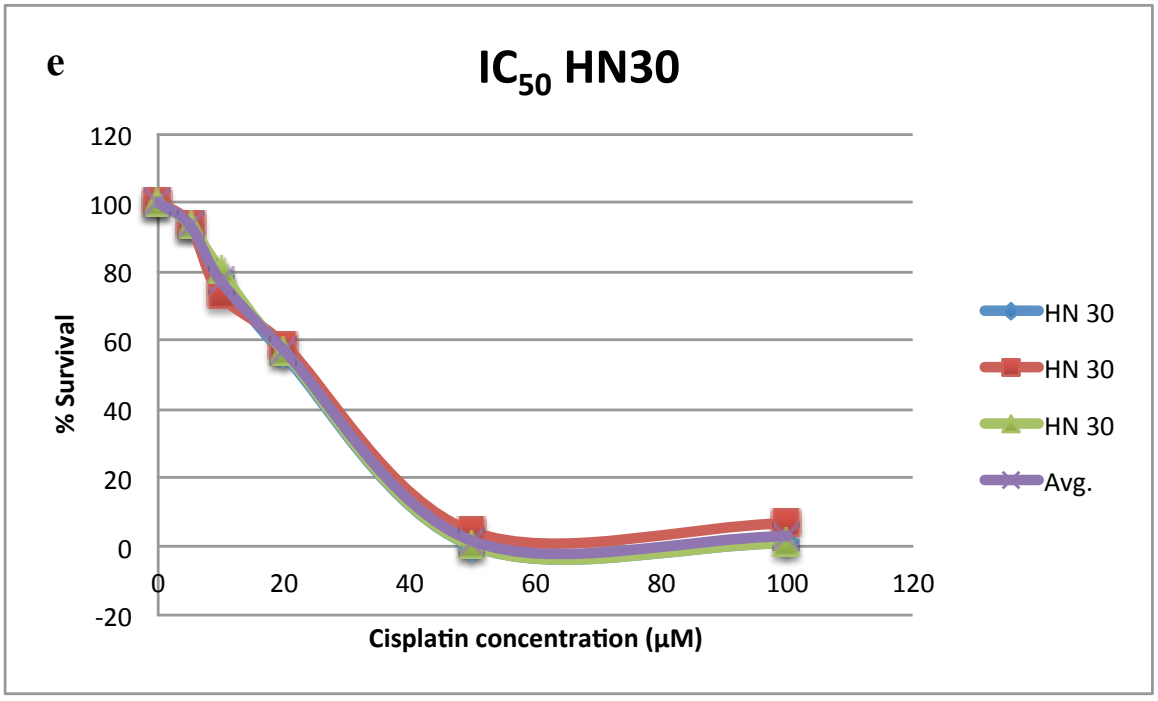
Table 2. IC_{50} values in HNSCC cell lines	
HNSCC cell lines	**Cisplatin sensitivity, IC_{50} (µM)**
HN4	75
HN12	63
HN22	50
HN8	50
HN30	21
HN31	20




b
IC50 HN12
120
100
80
60
40
20
0
-20
-40
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN 12
HN 12
HN 12
Avg.


c
IC50 HN22
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN22
HN22
HN22
Avg.


d
IC50 HN8
120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN 8
HN 8
HN 8
Avg.


e
IC50 HN30
120
100
80
60
40
20
0
-20
% Survival
0
20
40
60
80
100
120
Cisplatin concentration (µM)
HN 30
HN 30
HN 30
Avg.

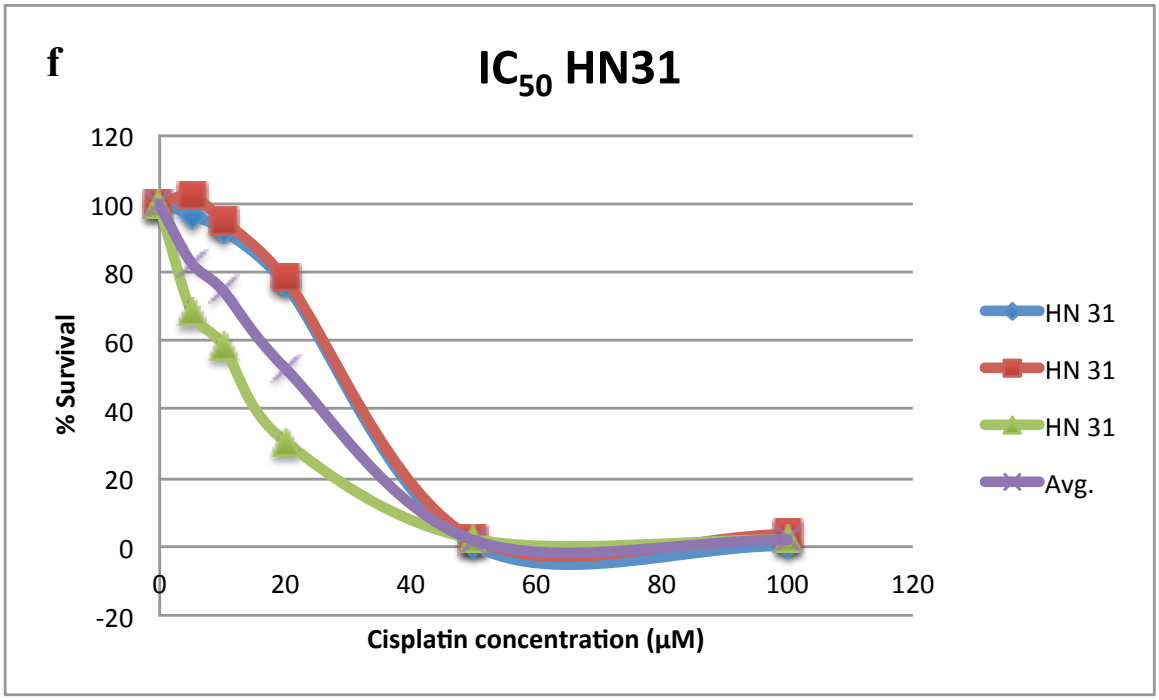


Figure 8: Cell viability determined by WST-1 assays. HNSCC cell lines were treated with various concentrations of cisplatin for 48 hours. (**a**) HN4 (**b**) HN12 (**c**) HN22 (**d**) HN8 (**e**) HN30 and (**f**) HN31

5.4 The contribution of Noxa to cisplatin-induced cell death

To examine if cisplatin induces Noxa to cause cell death, we introduced short-hairpin RNA (shRNA) for Noxa to knock-down Noxa expression. As control, we introduced non-targeting shRNA. Both sh-control and sh-Noxa expressing HNSCC cells were seeded in a 6-well plates and 20 μM of cisplatin was added to each well. Cells were harvested in 6 different time points, which revealed different expression levels of cleaved-PARP, MCL-1, and Noxa. Cleaved-PARP is one of the proteins cleaved by caspase-3 when apoptosis is occurred, therefore it was used to represent the induction of apoptosis. Figure 9a and 9b show clear reduction of cleaved-PARP levels with decreased Noxa expression. This decrease of cleaved-PARP expression suggests that Noxa is contributing to apoptosis by cisplatin in both HN30 and HN31 cells. The results also suggest the existence of Noxa-independent mechanisms to stimulate apoptosis.

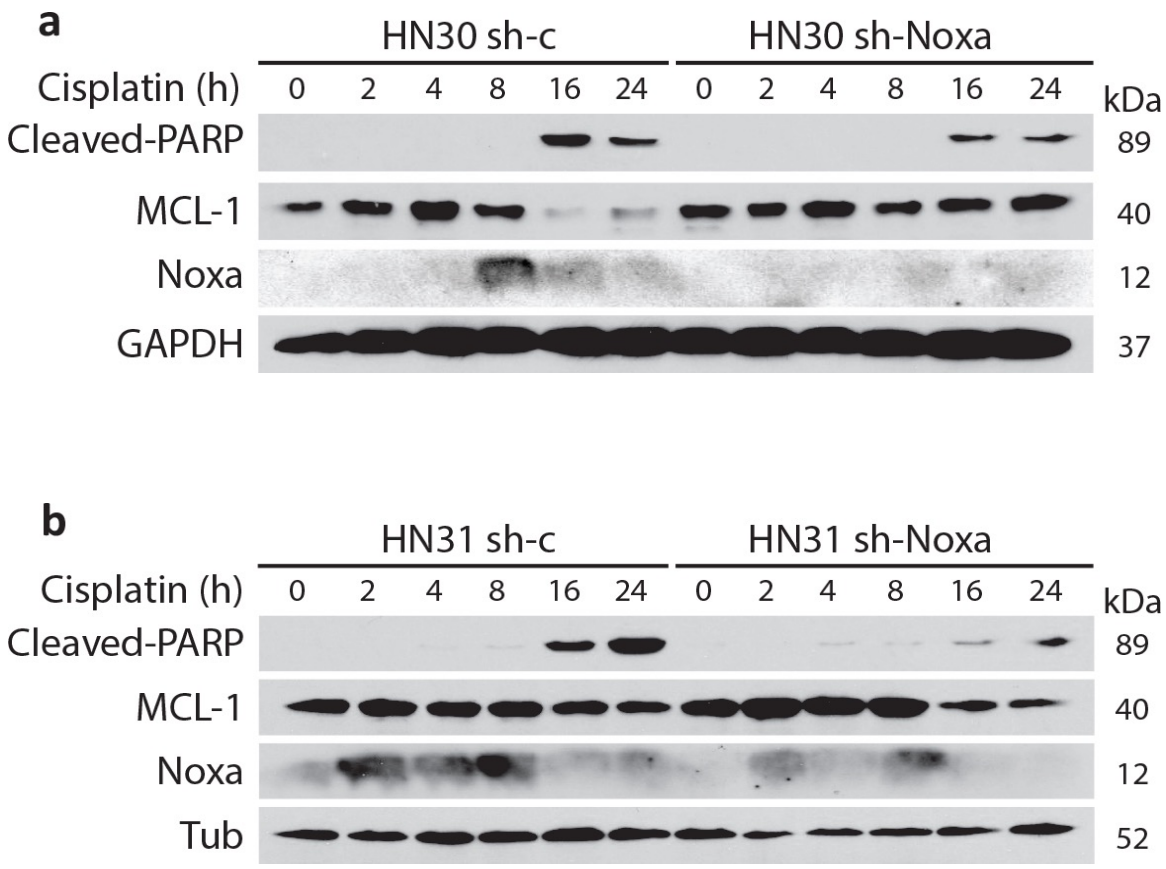


Figure 9: The induction of Noxa is required for cisplatin-induced apoptosis in HNSCC cells. (**a**) HN30 and (**b**) HN31 cells were infected with lentivirus-encoding shRNA for non-targeting control or Noxa. Cells were then treated with cisplatin (20 μM) for the indicated times and equal amounts of total extracts were loaded to immunoblot analysis with indicated antibodies.

5.5 The contribution of p53 to cisplatin-induced cell death through Noxa-independent pathway

p53 is activated by DNA damaging agents such as cisplatin. Noxa is known as a p53 target gene. Thus, to examine whether Noxa-dependent apoptosis induced by cisplatin observed in Figure 9 is also p53-dependent, we introduced shRNA for p53 in HN30 cells to knock-down p53 expression. Both sh-control and sh-p53 expressing HN30 cells were seeded in a 6-well plates and cells were treated with 20 μM of cisplatin. The level of cleaved-PARP was decreased in HN30 sh-p53 cells, whereas the induced level of Noxa was not much changed (Figure 10). This result suggests that cisplatin induces p53-dependent/Noxa-independent pathway to stimulate apoptosis and that Noxa can be induced independent of p53.

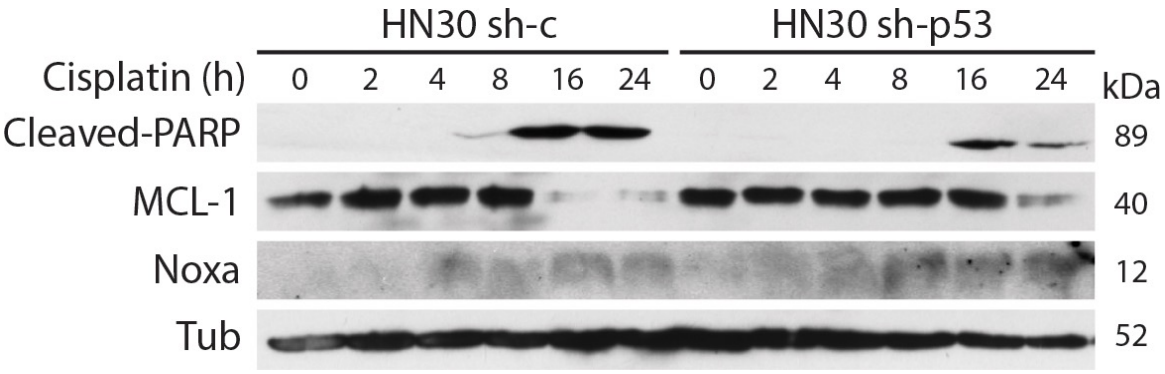
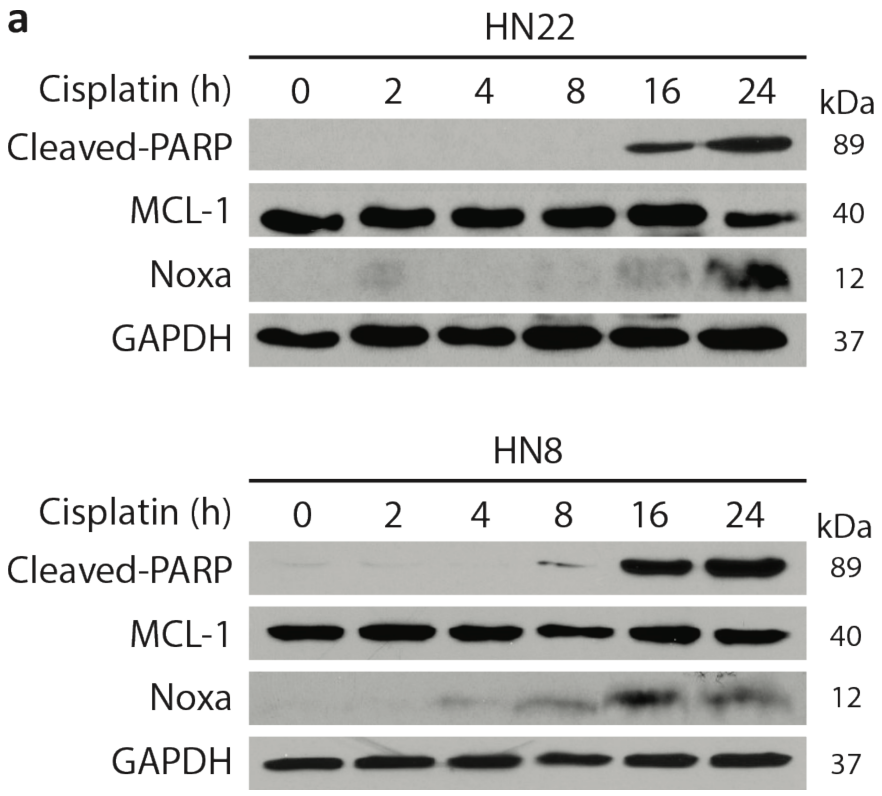


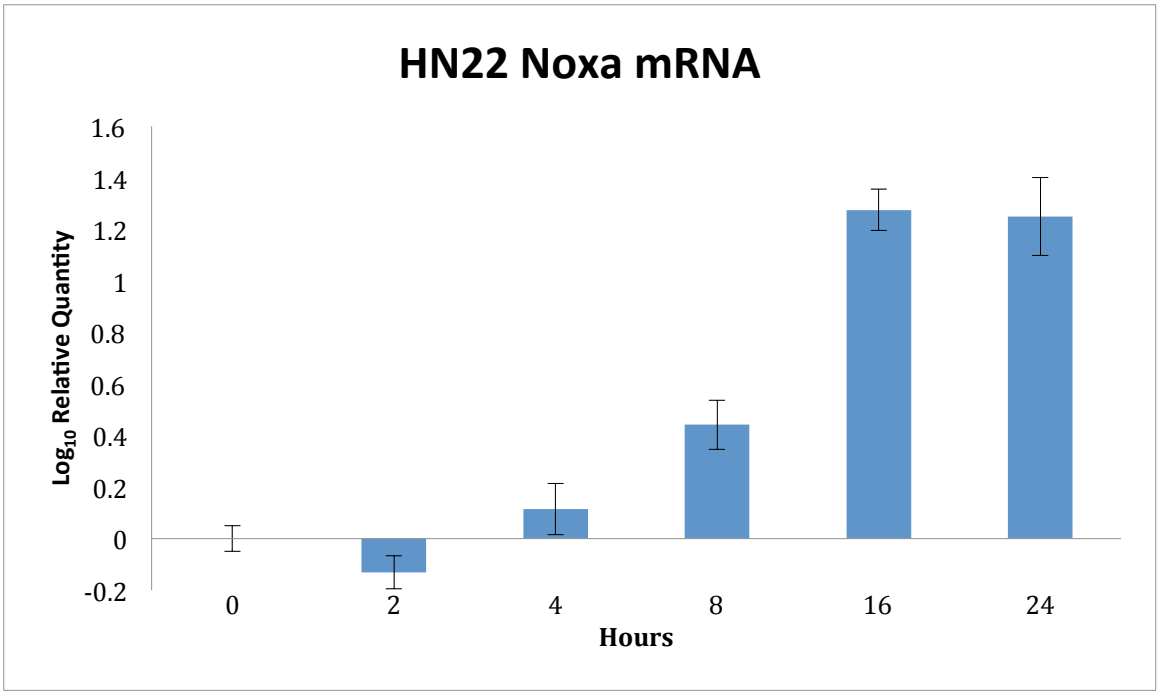
Figure 10: p53 contributes to cisplatin-induced apoptosis, but not to Noxa induction. Cells were infected with lentivirus-encoding shRNA for non-targeting control or p53. Cells were then treated with cisplatin (20 μM) for the indicated times and equal amounts of total extracts were loaded to immunoblot analysis with indicated antibodies.

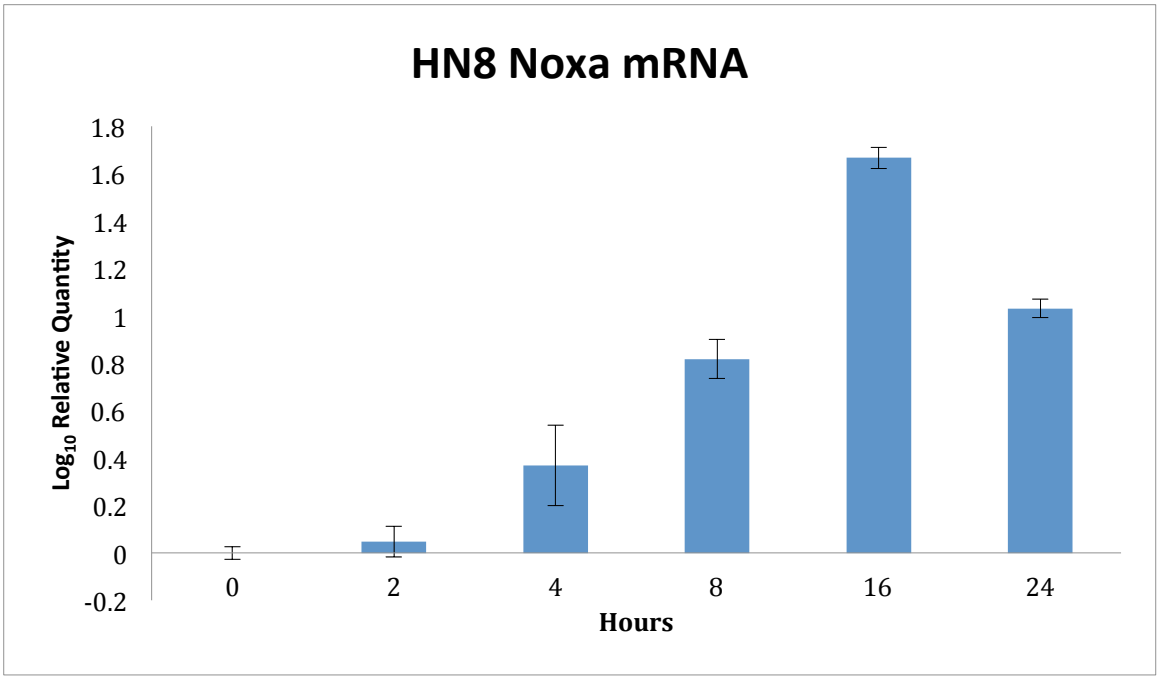
5.6 Noxa induction with cisplatin in p53 null cells

In order to examine the contribution of Noxa in cisplatin-induced apoptosis in p53-independent manner, we introduced shRNA for Noxa in p53-null HN22 and HN8 cells to knock-down Noxa expression. With IC_{50} results from WST-1 assay (Figure 8), 50 μM of cisplatin was used to treat HN22 and HN8 cell lines expressing sh-control and sh-noxa (Figure 11). Noxa is known to be the target gene of p53 [24], however, the immunoblot results in Figure 11a clearly showed the induction of Noxa with cisplatin treatment in both HN22 and HN8 p53-deleted cell lines. Figure 11b is showing mRNA levels of Noxa in HN22 and HN8 cells after cisplatin treatment. This data shows that cisplatin treatment induces Noxa by regulating transcription to increase the protein level. Down-regulation of Noxa reduced cisplatin-induced PARP-cleavage in HN22 cells (Figure 11c). These results suggest that Noxa can be induced by cisplatin with p53-independent pathways and consequently stimulates apoptosis.



b


HN22 Noxa mRNA
Log10 Relative Quantity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
2
4
8
16
24
Hours


HN8 Noxa mRNA
Log10 Relative Quantity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
2
4
8
16
24
Hours

c

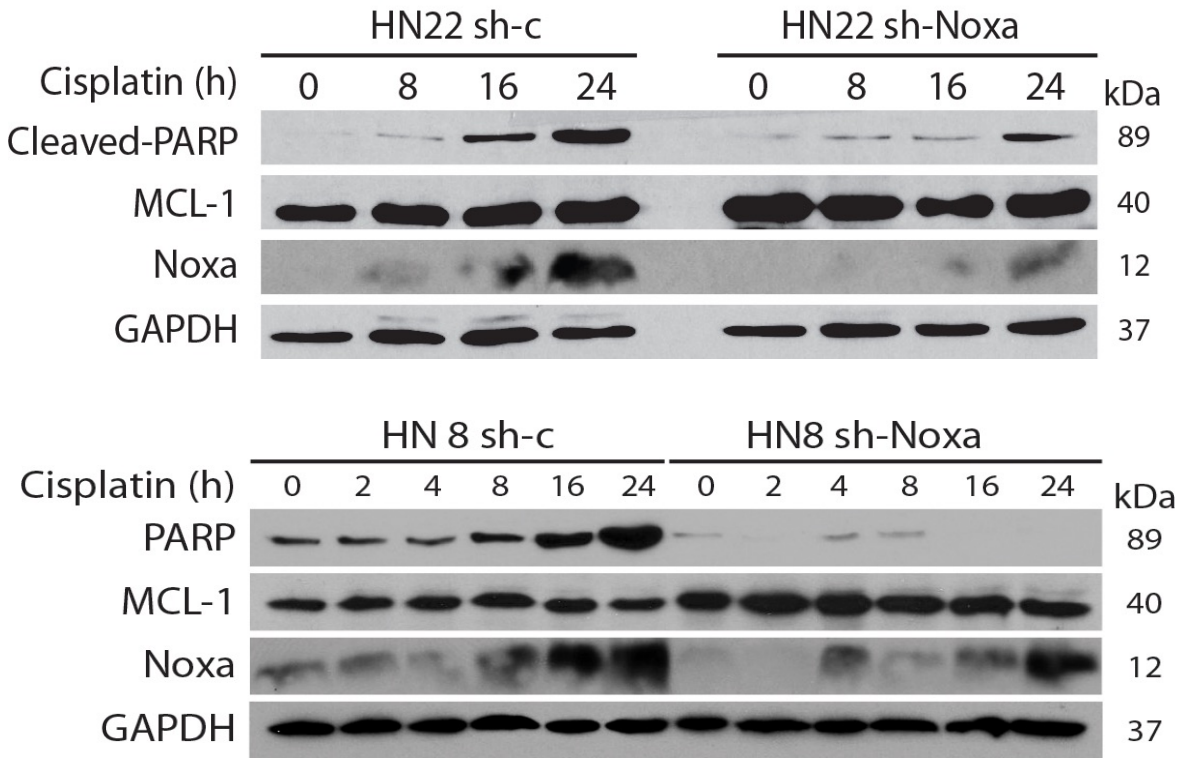


Figure 11: Noxa induction with cisplatin in p53-deleted cell lines. (**a**) HN22 and HN8 cells were treated with 50 μM of cisplatin for the indicated times. (**b)** Cisplatin-treated HN22 and HN8 cells were harvested at the indicated times and mRNA levels of Noxa were measured by qRT-PCR. (**c**) HN22 and HN8 Cells were infected with lentivirus-encoding shRNA for non-targeting control or Noxa. Cells were then treated with cisplatin (50 μM) for the indicated times and equal amounts of total extracts were loaded to immunoblot analysis with indicated antibodies.

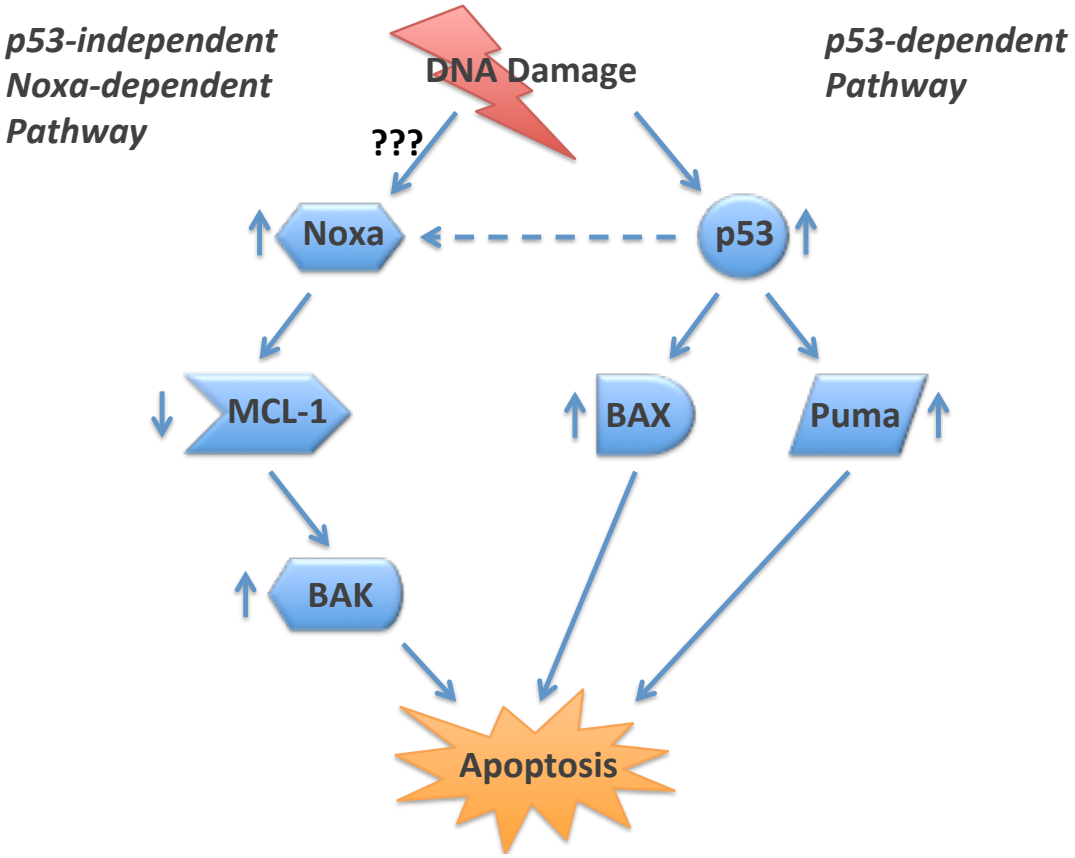


Figure 12: Schematics of p53-dependent/ Noxa dependent and p53-independent/ Noxa dependent pathways.

5.7 Overexpression of Noxa in HNSCC cells induces cell death

Noxa is known to sequester MCL-1 to be degraded, allowing the effector protein BAK to be released and resulting in cell death. Thus, we wanted to investigate if up-regulation of Noxa in HNSCC cells can induce apoptosis. We introduced Noxa cDNA using lentivirus-mediated transfection and a vector alone as control. The interaction of Noxa and MCL-1 was confirmed by immunoprecipitation followed by immunoblot analyses (Figure 13). We observed the increase of cleaved-PARP levels in all cell lines tested (Figure 14), suggesting that Noxa overexpression is sufficient to induce apoptosis in HNSCC cells regardless of the *p53* gene status. However, the amount of MCL-1 seemed to be differently by Noxa overexpression depending on the p53 status. HN4 and HN12 with truncated p53 had relatively similar amount of MCL-1 expression with or without Noxa overexpression. In contrast, p53-null HN22 and HN8 increased MCL-1 expression. These results suggest that a clear induction of cleaved-PARP with Noxa overexpression can be detected even though MCL-1 is not degraded by Noxa expression.

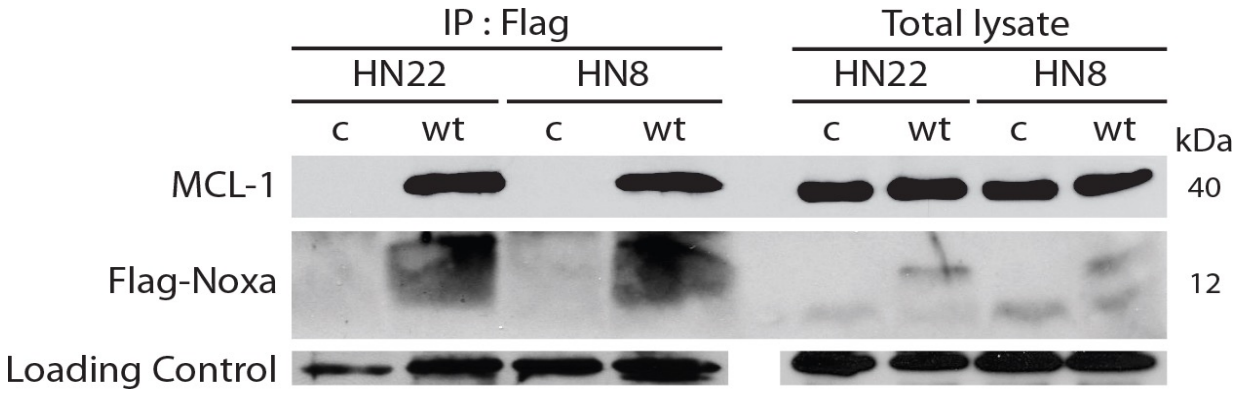


Figure 13: Noxa and MCL-1 interaction in HN22 and HN8 cells. Overexpression of Flag tagged-Noxa shows strong interaction with MCL-1 in both cell lines. Immunoprecipitation was done with the agarose beads conjugated with anti-Flag antibodies to capture Flag-Noxa. Total lysate shows MCL-1 and Noxa expression to compare with the IP. GAPDH is used as the loading control for the total lysate and expression level of the heavy chain from the agarose beads are used as the loading control for IP.

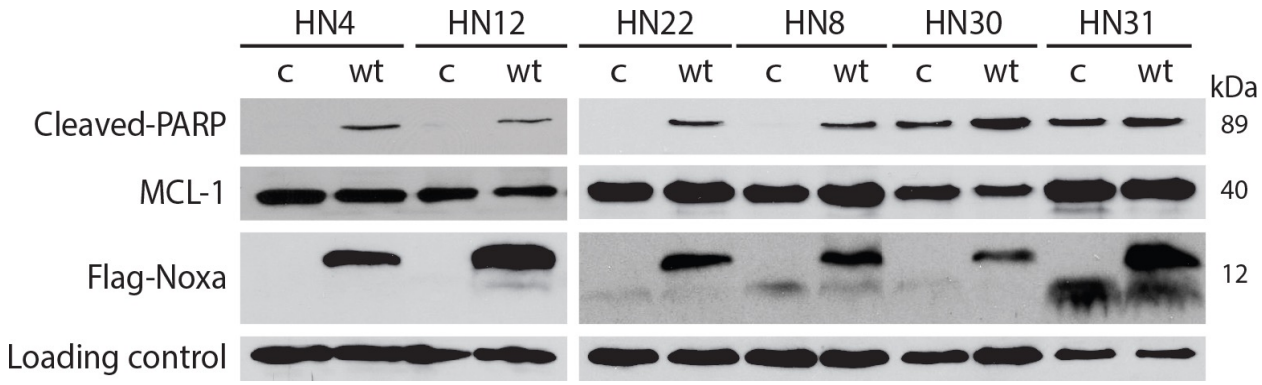


Figure 14: Apoptosis induced by Noxa overexpression in HN4, HN12, HN22, HN8, HN30, and HN31. HNSCC cells were infected with lentivirus-encoding Flag-tagged Noxa cDNA or vector alone as control for 24 hour. Equal amounts of total extracts were added to immunoblot analysis with indicated antibodies. The loading control for HN4 and HN12 were done with GAPDH, whereas HN22, HN8, HN30, and HN31 were done with alpha-tubulin. [C = control, wt = wild-type (overexpression) Noxa]

DISCUSSION AND FUTURE DIRECTIONS

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

When DNA is damaged by cisplatin, p53 is induced to prevent DNA replication or to stimulate apoptosis when DNA cannot be repaired. The IC_{50} results demonstrated that cell lines with wild-type p53 (HN30 and HN31) had higher sensitivity to cisplatin compared to cell lines with p53 deletion or non-functional p53 (Figure 6b and Table 2). From these results we can speculate that cisplatin largely induces p53-dependent pathway to stimulate cell death, however with increased cisplatin concentration, p53-deleted cells are able to induce cell death as well. This might suggest the existence of a p53-independent pathway(s) (Figure 12).

6.2 Knock-down of Noxa decreased cleaved-PARP expression with cisplatin treatment

A BH3-only protein, Noxa is known as a target gene for p53 [24]. We observed that there was decrease in cleaved-PARP expression and stabilization of MCL-1 in HN30 sh-Noxa, indicating that cisplatin-induced Noxa expression is required to stimulate apoptosis. Unlike HN30 sh-Noxa (Figure 9a), HN31 sh-Noxa (Figure 9b) still showed the reduction of MCL-1 expression in 16 and 24 hours after the treatment. Since endogenous Noxa expression in HN31 was much greater than HN30 (Figure 6a), the efficiency of

Noxa knock-down was not enough to decrease its expression. Noxa expression in 2, 4, and 8 hour period in HN31 sh-Noxa (Figure 9b) is clearly visible, therefore, this residual Noxa is likely to bind to MCL-1 and induce degradation. With overall results, we speculate that cisplatin can induce Noxa without p53, which could take longer time to induce cell death.

6.3 Knock-down of p53 decreased cleaved-PARP expressions with cisplatin treatment

To further investigate the pathways for the induction of cell death by cisplatin, we knocked-down p53 to examine the effects of cleaved-PARP, MCL-1, and Noxa. The result showed clear induction of Noxa expression in both HN30 sh-control and sh-p53 (Figure 10), but decrease in cleaved-PARP expression. Decrease in cleaved-PARP expression suggests that cisplatin induces a p53-dependent/Noxa-independent pathway(s). This result also suggests that Noxa can be induced not only by p53, but also by other mechanisms. However, we cannot conclude just by this result that p53-dependent pathway is the main pathway for cisplatin-induced cell death.

6.4 Noxa induction with cisplatin treatment in p53-deleted HNSCC cell lines

To analyze if Noxa expression can be seen in p53-deleted HN22 and HN8 cells by cisplatin, Western blots were performed with parental cells and with Noxa knock-down cells. The results (Figure 11a) clearly show the induction of Noxa in both HN22 and HN8 parental cells. This data confirms that Noxa can be induced by a p53-independent pathway(s). Figure 11b suggests that because the Noxa mRNA levels are increasing, its

induction is regulated by transcription, not by post-transcription protein stabilization. This data also supports that Noxa can be induced without p53.

Compared to p53 wild-type cells, these p53-deleted cells have more stable MCL-1 expressions even with increased Noxa expression (Figure 11 and 14), suggesting that there might be a p53 target gene for a specific E3-ligase to ubiquitinate MCL-1. p53 could also transcribe a target gene required for the MCL-1 localization at the outer membrane of mitochondria, which could enhance the stabilization of MCL-1.

6.5 Noxa overexpression can induce cell death regardless of p53 status

If exogenous Noxa expression can induce cell death, the status of p53 would not be prominent. Thus, we infected with lentivirus-encoding Flag-tagged Noxa cDNA to the HNSCC cell lines. Western blot results showed the increase of cleaved-PARP in all cell lines tested (Figure 14), suggesting that Noxa overexpression can induce apoptosis without consideration of p53 status. Because Noxa is downstream of p53, these results suggest that overexpression of Noxa is capable of inducing p53-independent/Noxa-dependent pathway to increase apoptosis. This finding could be applicable as a potential therapeutic strategy to treat head and neck cancer.

6.6 Future directions

More investigations should be conducted to elucidate the activation of p53-independent/Noxa-dependent cell death and figure out what could be inducing the expression of Noxa. MCL-1 stabilization in p53-deleted cells should also be thoroughly

investigated. Findings of other mechanisms that might inhibit MCL-1 ubiquitination or phosphorylation in p53 deleted cells could help to unravel more on how Noxa/MCL-1 proteins interactions are regulated.

LITERATURE CITED

LITERATURE CITED

1. American Cancer Society (2015). *Cancer Facts and Figures 2015.* Atlanta, GA: American Cancer Society. Retrieved March 05, 2015.
2. Viviana P. Lutzky. D. J et al. 2008. Biomarkers for Cancers of the Head and Neck. Clinical Medicine: Ear, Nose and Throat, 1,5-15.
3. King T. Agulnik. M. **2010**. Head and Neck Cancer: Changing Epidemiology and Public Health Implications. *Oncology Journal*: Head & Neck Cancer.
4. Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. *International Journal of Cancer* **2008**; 122(1):155-164.
5. Hashibe M. Boffetta P, Zaridze D, et al. Evidence for an important role of alcohol- and aldehyde- metabolizing genes in cancers of the upper aerodigestive tract. *Cancer Epidemiology, Biomarkers and Prevention* **2006**; 15(4):696-703.
6. Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Journal of the National Cancer Institute* **2007**; 99(10):777–789.
7. Boffetta P, Hecht S, Gray N, Gupta P, Straif K. Smokeless tobacco and cancer. *The Lancet Oncology* **2008**; 9(7):667–675.

8. Blot WJ, McLaughlin JK, Winn DM, et al. Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Research* **1988**; 48(11):3282–3287
9. Hashibe M, Brennan P, Chuang SC, et al. Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiology, Biomarkers and Prevention* **2009**; 18(2):541–550.
10. Zeka A, Gore R, Kriebel D. 2003. Effects of alcohol and tobacco on aerodigestive cancer risks: a meta-regression analysis. *Cancer Causes Control*, **14**:897-906
11. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of Clinical Oncology* **2011**; 29(32):4294-4301.
12. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: *A Cancer Journal for Clinicians* **2010**; 60(5):277-300
13. Poklar N, Pilch DS, Lippard SJ, Redding EA, Dunham SU, Breslauer KJ (July 1996). "Influence of cisplatin intrastrand crosslinking on the conformation, thermal stability, and energetics of a 20-mer DNA duplex" *Proc. Natl. Acad. Sci. U.S.A.* **93**(15):7606-11.
14. Rudd GN, Hartley JA, Souhami RL (1995). "Persistence of cisplatin-induced DNA interstrand crosslinking in peripheral blood mononuclear cells from elderly and young indiciduals" *Cancer Chemother. Pharmacol.* **35**(4):323-6
15. Function of Cisplatin (www.cisplatin.org/function.htm)

16. Jayaprakash V, Reid M. Hatton E, et al. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985-2010. *Oral Oncology* ***2011***; 47(11):1048-1054.

17. Patel AS, Lin L, Geyer A et al. (2012). Eickelberg, Oliver, ed. "Autophagy in idiopathic pulmonary fibrosis" *PLoS ONE* 7(7):e41394.

18. Wajant H. The Fas signaling pathway: more than a paradigm. *Science.* **2002**, 296(5573): 1635-1636.

19. Youle RJ, Strasser A. The BCL-2 protein family: opposing activites that mediate cell death. *Nat Rev Mol Cell Biol.* **2008**, 9:47-59.

20. Kozopas, K. M. et al., (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proceedings of National Academy of Sciences of United States of America*. **1993**, 90,3516-3520.

21. Gomez-Bougie P, Menoret E, Juin P, et al. Noxa controls Mule-dependent Mcl-1 ubiquitination through the regulation of the Mcl-1/USP9X interaction. *Biochem and Biophys Resear Comm.* **2011**, 413: 460-464

22. Gelinas C, White E. BH3-only proteins in control: specificity regulates MCL-1 and BAK mediated apoptosis. *Genes Dev.* **2005,** 19(11): 1263-8

23. Ploner C, Kofler R, Villunger A. Noxa: at the tip of the balance between life and death. *Oncogene.* **2009,** 27, S84-S92

24. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-Only Member of the Bcl-2 Family and Candidate Mediator of p53-Induced Apoptosis. *Science*. **2000,** 288(5468): 1053-1058

25. Thomas L, Lam C, Edwards S. Mcl-1; the molecular regulation of protein function. *FEBS Letters.* **2010,** 584(14): 2981-2989

26. Wang D. and Lippard SJ. Cellular processing of platinum anticancer drugs. *Nature Review Drug Discovery 4.* **2005,** 307-320

27. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. **2008,** 9:47-59.

28. Yeudall A, Jakus J, Ensley J, Robbins KC. Functional Characterization of p53 Molecules Expressed in Human Squamous Cell Carcinomas of the Head and Neck. *Molecular carcinogenesis*. **1997,** 18:89-96

29. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher J, Adams J, and Huang D. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-x_L, but not Bcl-2, until displaced by BH3-only proteins. *Genes & Development.* **2005,** 19:1294-1305

30. Hsu YT, Youle RJ. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *The journal of biological chemistry.***1998,** 273:10777-10783

31. Naik E, Michalak EM, Villunger A, Adams JM, and Strasser A. Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. *The Journal of Cell Biology.* **2007,** 176:415-424

32. Henshall D. and Engel T. Contribution apoptosis-associated signaling pathways to epileptogenesis: lesson from Bcl-2 family knockouts. *Front. Cell. Neurosci.* **2013,** 00110

33. Weinberg, Robert A. “Chapter 9: p53 and Apoptosis.” *The Biology of Cancer.* 2nd Edition ed. New York and London: Garland Science, **2014,** 331-389. Print.

34. Nakajima W, Hicks MA, Tanaka N, Krystal GW, and Harada H. Noxa determines localization and stability of MCL-1 and consequently ABT-737 sensitivity in small cell lung cancer. *Cell Death and Disease*. **2014,** 5, e1052

35. Green DR. Means to an End: Apoptosis and Other Cell Death Mechanisms. New York: Cold Spring Harbor Laboratory Press, **2011.** Print.

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