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Mechanisms of Apoptosis Induced by Actinomycin D in Aerodigestive Tract Cancers

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MECHANISMS OF APOPTOSIS INDUCED BY ACTINOMYCIN D IN AERODIGESTIVE TRACT CANCERS

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APPROVAL OF THESIS

We, the faculty supervising the work of Adeoluwa Ayodeji Adeluola, affirm that the thesis, *Mechanisms of Apoptosis induced by Actinomycin D in Aerodigestive Tract Cancers*, meets the high academic standards for original scholarship and creative work established by the Master of Sciences in Pharmaceutical Sciences Program and the Marshall University School of Pharmacy. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABSTRACT

Upper aerodigestive tract cancers including cancers of the oral cavity, pharynx, larynx, esophagus, and lungs are the most prevalent cancers and leading causes of cancer-related deaths. Collectively, over 300,000 new cases and 146,500 deaths are projected within the US in the year 2021. Drug-associated toxicities, as well as resistance to therapy (intrinsic and acquired), are big challenges for successfully treating these cancers. Recent studies have shown that combining low-dose actinomycin D with existing therapies is a promising strategy to reduce toxicity (cyclotherapy) and to overcome resistance. The development of these treatment strategies however requires an understanding of the molecular mechanisms of the antitumor activity of actinomycin D as well as that of drug resistance. This study evaluated the mechanism of actinomycin D-induced apoptosis and its effects on p53 signaling pathway in aerodigestive tract cancers using in vitro models of lung and head and neck cancer. We determined the IC_{50} of actinomycin D in a range of aerodigestive tract cancer cell lines using the SRB assay at 72 h which spanned between 0.02nM -2.96nM. Subsequently, we measured apoptosis by using the Annexin V-PE staining. FlowJo was used to quantify apoptosis. Cleavage of PARP and caspase 3 was used to confirm apoptosis. We found that actinomycin D induced apoptosis in all cell lines tested, but the sensitivity varies between cell lines. Mechanistic studies revealed that actinomycin D time- and dose-dependently increased the expression of p53 and its downstream targets p21 and PUMA in cells with wild-type p53. Consequently, we explored the role of p53 in actinomycin D-induced apoptosis by ablating the expression of p53 using p53 shRNA. Interestingly, the ablation of p53 decreased the expression of p21 and PUMA in A549 cell line and p21 in H460 cell line. More importantly, ablation of p53 significantly protected cells from actinomycin D-induced apoptosis. Furthermore, we explored the mechanism of apoptosis in cell lines lacking wild type p53 and focused on the expression of p73,

p21, and PUMA. Through the completion of these experiments, we can conclude that actinomycin D exerts apoptosis via p53-dependent and –independent mechanisms. These results will guide future combinatorial studies involving actinomycin D.

CHAPTER 1

INTRODUCTION

Background

Next to cardiovascular diseases, cancer is the second leading cause of death globally and in the United States of America (CDC, 2019). Aerodigestive tract cancers include tumors that develop from the tissues of the respiratory and the upper digestive tract, which include the lungs, bronchi, larynx, pharynx, oral cavity, and esophagus.

In 2020, lung cancer was the second most common cancer behind breast cancer, but the leading cause of tumor-related deaths amongst men and women of all ages around the world (2.2 million new cases and 1.7 million deaths) (Globocan, 2020; Siegel et al., 2016). Based on the morphology of the cancer cells, lung cancer is classified into two subtypes, namely non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC). NSCLC can be further classified as adenocarcinoma, squamous cell carcinoma, and neuroendocrine large cell carcinoma based on the histology of the disease. Smoking of tobacco and tobacco-related products is arguably the most important risk factor for lung cancer (Malhotra et al., 2016).

On the other hand, head and neck cancer (HNC) includes all tumors originating from the oral cavity, pharyngeal region, and larynx. Collectively, they were the seventh most common cancer in the world in 2020 (930,000 new cases and 467,125 deaths) (Globocan, 2020). Unlike lung cancer, HNC is mainly of squamous histology and risk factors include tobacco smoking, alcoholism, betel nut chewing, and HPV infections. The American Cancer Society estimates that in 2021, aerodigestive tract cancers will cause about 150,000 deaths in the US (Siegel et al., 2021).

Recent advances in oncology research have given scientists a better understanding of disease initiation, progression, and management. There are different modalities available for the

treatment of malignant disease, which include surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy depending on the stage, histology, and presence or absence of driver mutations.

For lung cancer, surgery is often recommended for early-stage (stage I-II) NSCLC patients. These patients have a 5-year survival rate of 50-90% depending on the stage of the disease at the initiation of therapy (Goldstraw et al., 2016). If surgery is contra-indicated or refused, high-dose stereotactic body radiation can be recommended which has a 5-year tumor control rate of more than 85% (Timmerman et al., 2010). However, in locally advanced cases where surgery is not possible, radiotherapy coupled with doublet chemotherapy is recommended but has a 5-year survival of 15-20% (Aupérin et al., 2010). The presence of activating mutations informs the selection of targeted therapies that are specifically aimed at genetic abnormalities that drive tumor progression (Hirsch et al., 2017). For HNC, surgery is also indicated in early-stage disease, but in locally advanced tumors where surgical benefits are poor, concurrent chemoradiotherapy with high dose cisplatin is the treatment of choice as it provides a 6.5% absolute 5-year overall survival benefit over radiotherapy alone (Cramer et al., 2019; Oosting & Haddad, 2019). For patients with recurrent or metastatic cases, the standard first-line maintenance is concurrent cisplatin, 5-FU plus cetuximab, because cetuximab provides a 13% increase in response rate and improves overall survival compared to chemotherapy alone (Vermorken et al., 2008; Vermorken et al., 2007). Immunotherapies such as pembrolizumab and nivolumab are also beneficial, especially amongst patients who progressed after platinum-based therapy as it improved overall survival by up to 20% in phase III CheckMate 141 trial (Harrington et al., 2017; Oosting & Haddad, 2019). Immunotherapies activate the patient's immune system against cancerous diseases by blocking certain factors that weaken immune surveillance.

Since the introduction of combination therapy in the 1960s, this strategy has been the mainstay therapeutic approach for cancer management due to its mounting advantage over monotherapy (Frei et al., 1965). Combinations are often comprehensive and include drugs with distinct mechanisms attacking different pathways to increase efficacy. However, the war against cancerous disease is far from won. Most cancers become resistant after the initial response to therapy, which leads to relapse, poor prognosis, and survival. Recent research has studied new combination strategies aimed at overcoming resistance to cancer therapy. In a concept called drug repositioning, some studies have discovered the antitumor and synergistic potential of medications approved for noncancerous indications when combined with conventional chemotherapeutics (Bayat Mokhtari et al., 2017). Others have used older cytotoxic therapeutics obtained from nature like Actinomycin D at low doses to target cancer stem cells with less risk of adverse effects (Green et al., 2019).

Nature serves as an invaluable resource for human existence. Over the years, giant strides have been made to improve the diagnosis and treatment of diseases by seeking natural remedies within the environment, cancer is not an exception. Microbes and plants obtained from land and marine habitats are the primary sources of these products (Demain & Vaishnav, 2011). Some of the natural sources of anticancer agents include; animals, microbes, and plants in terrestrial and marine environments. Since 1940, more than two-thirds of the over 140 anti-cancer agents approved for human use have been obtained from natural sources. Between 1960 and 1982, over 180,000 microbe-derived natural products were screened by the NCI for anti-tumor effects. In 2000, more than 50% of clinical studies for anti-tumor agents involved natural products (Cragg & Newman, 2000).

Table 1: List of approved anticancer agents from nature. Adapted with modifications from (Khazir et al., 2014).

Drug Name	Natural Source	Mechanism of Action	Approved Indications	FDA Approval
Mitomycin C	*Streptomyces spp.*	DNA cross-linking	Gastric and Pancreatic cancer	1956
Vincristine	*Catharanthus rosea*	Anti-mitotic	Acute Leukemia	1963
Actinomycin D	*Streptomyces parvulus*	DNA intercalation; RNA synthesis inhibition	Wilms tumor, Gestational trophoblastic neoplasia, Ewing's sarcoma	1964
Vinblastine	*Catharanthus rosea*	Anti-mitotic	Testicular cancer, HNC, Hodgkin's disease, Kaposi's sarcoma	1965
Doxorubicin	*Streptomyces peucetius*	DNA damage and Topoisomerase II inhibition	Breast, ovary, prostate, gastric, liver cancers, HNC, and SCLC	1966
Daunomycin	*Streptomyces peucetius*	Topoisomerase II inhibition	Pediatric acute myelocytic leukemia	1966
Teniposide	*Podophyllum peltatum*	DNA polymerase inhibition	Acute lymphoblastic leukemia	1967
Bleomycin	*Steptomyces verticillus*	DNA damage	Squamous cell carcinoma, testicular carcinoma	1973
Estramustine		DNA Alkylation	Prostate carcinoma	1979
Etoposide	*Podophyllum hexandrum*	DNA alkylation	Testicular cancer, SCLC	1980
Vinorelbine	*Catharanthus rosea*	Topoisomerase II inhibition	NSCLC	1989
Paclitaxel	*Taxus brevifolia*	DNA damage	NSCLC, Breast and ovarian cancer	1993
Irinotecan	*Camptotheca acuminata*	DNA damage	Colorectal cancer	1994
Docetaxel	*Taxus brevifolia*	Topoisomerase II inhibition	Breast, gastric, and Prostate cancers, HNC, SCLC	1995
Topotecan	*Camptotheca acuminata*	Topoisomerase I inhibition	SCLC, Cervical, and ovarian cancers	1996

Actinomycin D is one such agent from nature. It comes from a class of compounds known as actinomycins, which were first isolated from bacteria *Streptomyces parvulus* by Waksman and Woodruff in 1940 (S. Waksman & H. B. Woodruff, 1940). This antibiotic was the first in its class to exhibit anti-tumor activity (Cortes et al., 2016). By the 1960s Actinomycin D was approved for the treatment of Wilms tumor, trophoblastic tumors, and Rhabdomyosarcoma (Hollstein, 1974). However, its use has been limited by significant toxicity, which has led to numerous studies trying to reduce its toxicity, improve its activity or find new indications. Recent reports have shown that low-dose actinomycin D in combination with other therapeutics could target cancer stem-like cells and potentially resolve resistance (Green et al., 2019). Also, low-dose actinomycin D has been actively studied in p53-based cyclotherapy approaches that protect normal cells from cytotoxic chemotherapeutics (Choong et al., 2009; Rao et al., 2013). However, the definite mechanism of action of actinomycin D especially in aerodigestive tract cancers remains elusive. Nevertheless, actinomycin D is known to exert its cytotoxic effects via genotoxic and ribosomal stress signals that can activate the p53 pathway. Thus, this study will investigate the effect of actinomycin D on p53-dependent and independent signaling in aerodigestive tract cancers. We expect that such a study will pave the way to use this drug in p53-based cyclotherapy to reduce the toxicities of existing drugs.

Aims and Objectives

Aim 1: To evaluate the cytotoxic effect of actinomycin D on aerodigestive tract cancer cells.

Objective 1: Conduct growth inhibitory assays and determine the IC_{50} of actinomycin D across a range of aerodigestive tract cancer cell lines using the SRB assay and CalcuSyn software.

Aim 2: To investigate actinomycin D-induced apoptosis in aerodigestive tract cancer cell lines.

Objective 2: Quantify dose-and-time-dependent apoptosis after treatment of aerodigestive tract cancer cell lines with actinomycin D using the Annexin V/7-AAD apoptosis assay and flow cytometry. Confirm apoptosis and define the mechanism of apoptosis induction by biochemical assay (Western Blots).

Aim 3: Determine the role and significance of p53 signaling in actinomycin D-induced apoptosis in aerodigestive tract cancer cell lines.

Objective 3: Compare apoptosis induction and p53 target genes expression by actinomycin D in p53 competent, mutant, and deficient cell lines.

CHAPTER 2

LITERATURE REVIEW

Aerodigestive Tract Cancers

Aerodigestive tract cancers form a group of malignancies that develop within tissues and organs of the respiratory and upper digestive system. The tissues implicated include the larynx, trachea, and lungs within the respiratory tract, and the lips and oral cavity, hypopharynx, nasopharynx, and oropharynx, and esophagus within the head and neck region of the upper digestive tract (Muir & Weiland, 1995).

Lung Cancer

Lung cancer is one of the most common cancers worldwide and the leading cause of death amongst men and women in the United States. Worldwide, almost 2 million new cases and more than 1.5 million deaths are recorded yearly with a 5-year survival rate below 20% (Hirsch et al., 2017). In the US, over 235,000 new cases and 131,880 deaths from lung cancer are projected for 2021, and the 5-year relative survival is 21.7% (NCI, 2021).

The rate of lung cancer cases within a population depends on demographic characteristics like age, gender, race, and socio-economic status. On a global scale, the trend in cases and mortality appear to shift from the western countries where more people are quitting smoking to lower and middle-income countries where smoking uptake is still rampant. In the United States, mortality is highest in males and people of color especially from mid-southern states of Mississippi, Kentucky, Arkansas, and Tennessee (Torre et al., 2016).

Based on the morphology of cells under the microscope, there are two types of lung cancer, NSCLC, and SCLC. NSCLC is further classified into adenocarcinoma, squamous cell carcinoma, and neuroendocrine large cell carcinoma based on the histological origin of the disease.

Meanwhile, the currently recognized histologic sub-classifications for SCLC are pure SCLC and combined SCLC. Combined SCLC refers to SCLCs that have at least 10% of larger cells that qualify as NSCLC cells (Raso et al., 2021; Travis, 2012). The therapy for lung cancer is generally rife with challenges like chemoresistance, but significant improvement in the characterization of the disease and personalization of care could improve treatment success in the future. For instance, the identification of molecular biomarkers like EGFR mutations and anaplastic lymphoma kinase-ALK translocations at the point of diagnosis would significantly inform the chemotherapeutic approach and improve treatment outcomes (Villalobos & Wistuba, 2017).

Epidemiology of Lung Cancer. In 1964, the US Surgeon General reported that men who were average smokers (10-20 cigarettes/day) had up to 10 times more risk of lung cancer than non-smoking men, heavy smokers (20 or more cigarettes/day) had up to 20 times more risk. Subsequently, public attitudes and policies towards cigarette smoking changed (Health, 1964). Before 1964, about 66% of adult males and 33% of adult females within the American population were active smokers. Over fifty years later in 2018, less than 15% of the US adult population were current smokers (Creamer et al., 2019). However, this varied between racial groups, gender, and socio-economic status. Smoking rates were higher amongst Indian Americans and native Alaskans, while it was lowest amongst Asian Americans. Also, smoking is prevalent among poorer and less educated populations (Schiller et al., 2012; Schwartz & Cote, 2016).

Today smoking is still the most important risk factor for lung cancer development as it increases the risk by up to 85% depending on exposure. Other environmental risk factors include; asbestos, radon, diesel, and ionizing radiation (Samet et al., 2009; Schwartz & Cote, 2016). The strong link between lung cancer and tobacco smoking is depicted by the similarity in the geographical distribution in lung cancer incidence and smoking culture around the world. For

instance, regions with a greater smoking culture like Central and Eastern Europe (53.5 per 100,000) and East Asia (50.4 per 100,000) have the highest incidence rates in men, while places like central and west Africa (2.0 and 1.7 per 100,000, respectively) have the lowest rates. Likewise, the geographic pattern of fatalities follows a similar trend to incidence rates due to high mortality (Mao et al., 2016).

Non-small cell lung cancer (NSCLC). Of the two forms of lung cancer, NSCLC has a higher prevalence with about 85% of all lung cancer cases being NSCLC. It is classified into three subtypes based on the histology of the malignancy. The three forms include; Adenocarcinoma (40-70%), Squamous cell carcinoma (20-30%), and neuroendocrine large cell carcinoma (10-15%) (Collins et al., 2007). Of the three subtypes, adenocarcinoma is the most common and least associated with smoking. It is highly metastatic at the early stage and common amongst never smokers with comorbid lung disease. On the contrary, squamous cell carcinoma undergoes late metastasis and is associated with symptoms like hemoptysis, post-obstructive pneumonia, and lobar collapse. Lastly, neuroendocrine large cell carcinoma is a rare form of NSCLC, and it is a poorly differentiated tumor, and like adenocarcinoma, it metastasizes early (Collins et al., 2007; Travis et al., 1995).

Early diagnosis and identification of driver mutation and histologic variants are essential for effective management especially in this era of targeted therapies with small molecule inhibitors and immunotherapy (Liu et al., 2017; Neal et al., 2014).

Staging. Following tissue diagnosis, patients are evaluated for metastatic spread to determine the stage of the disease. This evaluation is often achieved through physical examination, biochemical tests, and radiographic imaging such as computed tomography and positron emission tomography scans. NSCLC is staged based on a tumor-node-metastasis (TNM) system, which

classifies the cases into local (IA, Ib, IIA) locally advanced (IIB, IIIA, IIIB) and advanced (IIIB, IV) based on the size of the primary tumor, lymph node involvement, and metastasis to secondary sites (Collins et al., 2007; Travis et al., 2016).

Standard of Care (Early Stage). Surgical resection is the first-line recommendation for patients with stage I-II NSCLC. The outcomes and 5-year survival depend on the clinical or pathological stage of the disease. Stage IA (77-92%), stage IB (68%), stage IIA (60%), stage IIB (53%). Pathological stage IA (80-90%), stage IB (73%), stage IIA (65%), stage IIB (56%) (Hirsch et al., 2017; Travis et al., 2016; Vansteenkiste et al., 2014). Perioperative chemotherapy has been beneficial to patient survival especially stage IB-IIIA ("Preoperative chemotherapy for non-small-cell lung cancer: A systematic review and meta-analysis of individual participant data," 2014). In cases where surgery is contra-indicated high-dose stereo-static body radiation is an option for local control of tumor growth. Other non-operative first-line therapies include; radiofrequency ablation, standard radiotherapy, and chemotherapy. For patients with locally advanced tumors (stage IIIA & B) who cannot undergo surgical resection, it is recommended that they undergo a 6-week course of thoracic chemoradiation. The chemotherapy is usually a platinum-based drug (cisplatin or carboplatin) with a second drug dosed weekly or every 3-weeks (Aupérin et al., 2010; Curran et al., 2011; Hirsch et al., 2017).

Standard of Care (Advanced Stage). With advances in technology and pharmacogenetic research, it is evident that in advanced-stage disease, the primary tumor in many patients would have undergone some form of activating mutation that drives the disease. It is therefore pertinent to test for activating mutations using molecular biomarkers while considering the histology of the tumor (adenocarcinoma or squamous cell carcinoma) to inform a therapeutic approach. For

patients without driver mutations, platinum-based therapy remains the first-line treatment option (Hirsch et al., 2017).

EGFR Activating Mutation. EGFR activating mutations are common amongst lung cancer patients with studies reporting up to 40-55% prevalence in NSCLC especially amongst patients of Asian descent (Huang et al., 2004; Kosaka et al., 2004). Some of the popular demographic characteristics among patients with EGFR mutations include; East Asian, young age, female gender, adenocarcinoma, and absence of smoking history. Exon 19 deletion and Exon 21 substitutions (L858A) are common mutations that result in sensitivity to EGFR tyrosine kinase inhibitors [TKI] (Fujimoto & Wistuba, 2014). Gefitinib and erlotinib (first generation) and afatinib (second generation) were the initial set of small molecule inhibitors of EGFR tyrosine kinase approved by the FDA (Hammerman et al.; Solassol et al., 2019). However, the tumor often recovers and acquires resistance to initial tyrosine kinase inhibition either by secondary EGFR mutation, alternative activating mechanisms, or histological transformation. The gatekeeper mutation T790M is the usual culprit for secondary mutations and resistance after initial EGFR TKI sensitivity. Consequently, FDA and EMA approved Osimertinib, a third-generation TKI for patients with T790M mutation for whom initial TKI therapy had failed. In the absence of T790M mutation, platinum-based doublet chemotherapy remains the standard of care. Interestingly, EGFR C797S mutation, HER2, MAPK activation, and MET amplification are other forms of genetic changes that bypass the T790M mutation inhibited by Osimertinib (Hirsch et al., 2017). Even though there are about three dozen FDA-approved small molecule TKIs, secondary resistance is gradually becoming the norm. Further research is required to identify new strategies to overcome resistance (Roskoski, 2019).

ALK gene Rearrangements. Other than EGFR mutations, the discovery of a fusion gene consisting of the echinoderm microtubule-associated protein-like 4 (EML4) gene and the ALK gene has impacted the clinical outcome of the subset of NSCLC patients with this fusion oncogene (Soda et al., 2007). ALK mutations are present in about 3-8% of all NSCLC cases (Devarakonda et al., 2015). Most patients in this population are middle-aged men of Asian descent who were never smokers or light smokers with adenocarcinoma (Chatziandreou et al., 2015). ALK fusions usually occur in the absence of other driver mutations like EGFR, ROS1, and KRAS mutations. Crizotinib, ceritinib, alectinib are some of the inhibitors that have been developed for patients in this category. In 2011, the FDA granted crizotinib accelerated approval for the treatment of metastatic NSCLC with ALK rearrangements (Kazandjian et al., 2014). Interestingly, crizotinib can also inhibit ROS1, and MET (Gainor et al., 2013; Hirsch et al., 2017; Shaw et al., 2009).

TP53 Mutations. It is not surprising that the TP53 gene is the most frequently mutated in NSCLC irrespective of histology, occurring in about 32.5% of NSCLC tumors (Forbes et al., 2011; Goh et al., 2011). Some groups have classified these mutations to either be disruptive or non-disruptive. Disruptive in the sense that the mutation leads to a complete or almost complete loss of function of the mutant protein. Whereas non-disruptive mutations would retain some wildtype p53 functionality and in some cases, gain-of-function (Molina-Vila et al., 2014; Poeta et al., 2007). However, more studies are required to determine if these mutations are equally distributed or prevalent in certain histologic subtypes of lung adenocarcinoma.

Other Genetic Abnormalities in NSCLC. About 1-2% of all NSCLC cases have ROS1 gene rearrangement yielding up to nine variants of the protein. Most patients with this aberration have adenocarcinoma, are young females (median age: 50) who were never or light smokers. Crizotinib was approved by the FDA as an inhibitor for ROS1 positive patients. However, acquired

resistance has been observed through secondary mutations. This has inspired the investigation of other potential ROS1 inhibitors such as; ceritinib, entrectinib, lorlatinib, and cabozantinib (Bergethon et al., 2012; Hirsch et al., 2017). In 2019, FDA approved entrectinib in adult patients for ROS1-positive metastatic NSCLC. Emerging data have demonstrated efficacy of lorlatinib in ROS1-positive+ NSCLC patients, especially in crizotinib resistant cases (Shaw et al., 2019).

Similarly, RET fusion is observed in 1-2% of NSCLC cases, most of whom were never or light smokers with adenocarcinoma or adenosquamous carcinoma (Wang et al., 2012). Clinical studies for RET kinase inhibitors such as; apatinib, lenvatinib, cabozatinib, sorafenib, ponatinib, alectinib, vandetanib, and sunitinib are in progress (Califano et al., 2015; Hirsch et al., 2017). In 2020, FDA granted approval to selpercatinib and pralsetinib for RET-fusion positive NSCLC and thyroid cancer, and RET mutant medullary thyroid cancer (Markham, 2020).

Reports show that a splice site mutation at exon 14 of the MET receptor tyrosine kinase gene, was observed in 3-4% of lung adenocarcinomas. This mutation led to increased kinase activity, which was reduced by MET inhibition with crizotinib and cabozantinib (Hirsch et al., 2017; Paik et al., 2015). Crizotinib was originally developed as a MET inhibitor, but was subsequently approved for the treatment of ALK positive advanced or metastatic NSCLC patients (Kazandjian et al., 2014)

Overexpression and amplification of HER2 occur in 35% and 10% of all lung cancers, respectively. However, mutation of the HER2 gene is present in 2% of NSCLC cases usually in women with adenocarcinoma who were never smokers (Hirsch et al., 2017; Mazières et al., 2013). Recent studies have suggested the application of trastuzumab emtansine, an antibody-drug conjugate (ADC) that has benefited HER2+ breast cancer patients, as a treatment for HER2+ NSCLC in combination with other cytotoxic agents (Hotta et al., 2018; Peters et al., 2019).

In NSCLC, BRAF mutation is found in about 3-5% of the patients, usually among patients with adenocarcinoma who have a smoking history (Villalobos & Wistuba, 2017). The Val600Glu mutation is reported in about half of the cases. Such cases have shown good response to a combination of dabrafenib and trametinib (Dankner et al., 2018; Hirsch et al., 2017; Planchard et al., 2015) which were approved for patients with this mutation.

KRAS mutations are a common occurrence in lung adenocarcinomas (1 in 4 cases) usually among smokers of non-Asian descent. This mutation has been difficult to control, but some studies have reported positive responses with MEK inhibitors (trametinib and selumetinib) in combination with chemotherapy (Dearden et al., 2013; Hirsch et al., 2017; Jänne et al., 2013; Mazieres et al., 2013).

It is noteworthy that genomic aberrations are not limited to lung adenocarcinomas. There are reports of lung cancers of squamous histology with genomic aberrations such as mutations and amplification of FGFR, deregulation of the PI3K pathway, and inactivation of tumor suppressors TP53 and p16. However, studies are yet to prove if such alterations are significant to warrant a sub-population that could benefit from targeted therapies. This presents an opportunity for further research (Hirsch et al., 2017).

Immunotherapy for NSCLC. The initiation and progression of cancer are determined by a delicate interplay between the immune system and the genomic and/or molecular character of the cancer cells. The evasion of immune surveillance is an emerging hallmark of cancer. Efforts to develop a vaccine that activates the immune system against cancer cells have been relatively ineffective (Hirsch et al., 2017). However, the development of antibody therapy against inhibitory regulators of immune surveillance has shown positive results. Some of the modulators

that have been successfully inhibited with antibody therapy include the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the programmed death protein 1 pathway (PD1/ PD-L1).

CTLA-4 has an important role in curtailing immunologic response. This is achieved by a variety of mechanisms that ultimately leads to a decrease in the functionality of activated T lymphocytes (Postow et al., 2015). Ipilimumab was approved by the FDA in 2011 for the treatment of various tumors like melanoma and renal cell carcinoma (Vaddepally et al., 2020). In a phase II study involving NSCLC patients, the CTLA-4 inhibitor ipilimumab when combined with platinum-based carboplatin and paclitaxel offered comparative benefits in progression-free survival when compared with chemotherapy alone (Lynch et al., 2012). However, in a larger phase III study, the addition of ipilimumab to chemotherapy failed to improve overall survival in patients with advanced squamous NSCLC (Govindan et al., 2017). The CheckMate 9LA Trial tested the efficacy of PD-1 inhibitor nivolumab and CTLA-4 inhibitor ipilimumab combined with chemotherapy vs. chemotherapy alone in first-line NSCLC and based on the results of this trial, FDA approved the combination of ipilimumab and nivolumab plus chemotherapy as first-line therapy for patients with metastatic and recurrent NSCLC without EGFR or ALK driver mutations (Paz-Ares et al., 2021).

Like CTLA-4, PD1 expressed on the surface of T cells also thwarts T lymphocyte activity, but PD1 must bind to its ligands PDL1 and PDL2 (expressed on the surface of tumor cells, macrophages, dendritic cells, and T cells in the tumor micro-environment) to exert its inhibitory effects. Ligand-receptor interaction inhibits the kinase pathway that activates T lymphocytes (Han et al., 2020). Some antibodies that are currently approved for clinical use include; Nivolumab and pembrolizumab which target the PD-1 receptor and atezolizumab, durvalumab, and avelumab that target the PD-L1 ligand. The PD modulators seemed effective irrespective of histology or genomic

abnormalities, however, patients with smoking history and those with positive PD-L1 expression had better outcomes (Hirsch et al., 2017). Nivolumab has been approved by the FDA (SCC and non-SCC) and EMA (SCC) NSCLC patients as second-line after progression with chemotherapy. Also, the FDA approved pembrolizumab as second-line if more than 50% of tumor cells express PD-L1 (Hirsch et al., 2017). Currently, pembrolizumab has several indications for recurrent and metastatic NSCLC irrespective of PD-L1 status (Vaddepally et al., 2020).

Small Cell Lung Cancer (SCLC). Although SCLC occurs in only about 15% (about 200,000 cases) of newly diagnosed lung cancers, it is often a more aggressive disease with a higher metastatic potential. It is more common among elderly patients with heavy smoking history. Based on the Veteran's Administration (VA) lung classification scheme, SCLC is usually classified into limited and extensive stages for clinical trials and treatment decision purposes. In limited-stage (LS) SCLC, the tumor is confined to 1 hemithorax with or without nodal metastasis and can be safely and completely targeted by a radiation field. Extensive stage (ES) SCLC disease usually occurs in over 60% of new cases and cannot be safely targeted by a radiation field (Wang et al., 2019). There is also a tumor node and metastasis classification that groups cases based on solid tumor size, nodal metastasis, and presence or absence of distant metastasis (Goldstraw et al., 2016). Metastasis to the brain, adrenal glands, bone marrow, and liver are common occurrences. Notably, about 20% of new cases have brain metastasis at the time of diagnosis (Seute et al., 2008).

Concerning molecular abnormalities, most SCLC cases have alterations in the tumor suppressors RB1 and TP53. Also reports show that the most frequent alterations with targets include; PI3KCA (6%), RICTOR (10%), KIT (7%), EGFR (5%), KRAS (5%), PTEN (5%), MCL1 (4%), FGFR1 (4%) and BRCA2 (4%)(Ross et al., 2014). Although none of these have been

pursued clinically due to technological limitations to validate the aberrations, more than 50% of SCLC cases had at least one actionable alteration (Ross et al., 2014). A high risk of a mutation linked to tobacco carcinogens could explain why there has been little breakthrough in the management of the disease. For many years, etoposide and platinum analogs were the first-line approved therapy for SCLC, while topotecan was approved for recurrent disease (Hann & Rudin, 2008). But only 1 in 10 patients survived beyond the fifth year of diagnosis (Wang et al., 2019). In recent years, further research has led to the approval of newer agents like nivolumab, atezolizumab, and durvalumab combined with etoposide and platinum analogs.

Standard of Care for Limited-Stage SCLC. A combined approach of chemotherapy and radiotherapy is the mainstay first-line management for patients with LS-SCLC. There is substantial evidence that justifies the combination strategy compared to chemotherapy alone (Pignon et al., 1992; Warde & Payne, 1992). Systemic chemotherapy includes administration of cisplatin and etoposide alongside thoracic irradiation. The timely administration and duration of radiotherapy are crucial to determining the overall survival benefit to the patient (Fried et al., 2004; Pijls-Johannesma et al., 2007; Turrisi et al., 1999). The current advice is to administer a total of 45 Gy radiation (1.5 Gy twice daily) during 4-6 cycles of doublet chemotherapy (etoposide and cisplatin). There is also evidence that prophylactic cranial irradiation decreases the risk of cranial metastasis by 50%. For patients with bulky intrathoracic disease, the neoadjuvant approach may be considered to reduce the tumor size for safe and effective radiotherapy (Board, 2002; Wang et al., 2019).

Standard of Care for Extensive-Stage SCLC. Since the 1980s, the standard of care for ES-SCLC has been combination chemotherapy of etoposide and platinum-based (cisplatin or carboplatin) drugs. However, resistance to chemotherapy often ensues after an initial response to

chemotherapy. In Japan, some studies have replaced etoposide with irinotecan in combination with cisplatin. The results show comparable efficacy with no significant advantage of irinotecan over etoposide therapy (Lara et al., 2009; Noda et al., 2002). More so, the evidence that adding an immune checkpoint inhibitor atezolizumab to the combination chemo offered additional overall survival advantage, made a case for future chemoimmunotherapy in this sub-population (Horn et al., 2018; Wang et al., 2019). This led to the approvals of nivolumab, atezolizumab, and durvalumab as immunotherapies for ES-SCLC (Horn et al., 2018; Paz-Ares et al., 2019; Ready et al., 2020).

Prophylactic Cranial Irradiation (PCI) is recommended for patients who have responded positively to platinum-based therapy. This decreases the risk of brain metastasis but comes with the risk of neurocognitive defects that may impact the quality of life of the patients. Consequently, PCI is not considered a standard of care for ES-SCLC. However, it is administered on a case-by-case basis after careful risk-benefit consideration and discussion with the patient (Wang et al., 2019).

For refractory cases of SCLC, topotecan is the most common second-line drug of choice (Kalemkerian et al., 2013). Amrubicin is also approved in Japan for the same indication (von Pawel et al., 2014). It is noteworthy that many genomic aberrations that could yield actionable targets are currently being investigated in clinical studies. Aberrations that impact cell cycle progression, NOTCH signaling, tyrosine kinase signaling, and epigenetic modifications are some of the areas being studied (Wang et al., 2019).

Immunotherapy for SCLC. The fact that SCLC is by far a cancer of smokers also means there is a high rate of somatic mutations resulting from tobacco carcinogens. These cases could benefit from immunological activation against cancer cells. Many studies are currently

investigating the effects of PD1 (Pembrolizumab & Nivolumab) and PDL1 inhibitors (Atezolizumab & Durvalumab) in SCLC as single therapy (Ott et al., 2017), or in combination with anti-CTLA-4 inhibitors (ipilimumab) (Antonia et al., 2016). Based on positive reports from ongoing studies, Nivolumab-ipilimumab is recommended as a second-line therapy option for ES-SCLC, while Nivolumab was approved by the FDA as the third line for metastatic SCLC disease (Antonia et al., 2016).

Additionally, there are many cytotoxic-antibody drug conjugates currently being investigated in clinical studies. This would increase the efficacy of targeting and concentrating cytotoxic drugs at the tumor sites. Some examples include; Etirinotecan pegol, Dinutuximab, and Lurbinectedin (Wang et al., 2019).

Head and Neck Squamous Cell Carcinoma (HNSCC)

HNSCC is a heterogeneous group of malignancies originating from the epithelial cells of the oral cavity, pharynx (oro, naso, hypo), and larynx. Together, they rank seventh among the most common cancers worldwide, accounting for over 600,000 new cases every year (Ferlay et al., 2019; Globocan, 2020; Johnson et al., 2020). At the point of diagnosis, most patients have a locally advanced tumor that would have spread to the lymph nodes. Consequently, treatment approaches usually combine surgical, radiotherapy, and chemotherapy options to achieve the best outcomes for the patient. About 50% of therapeutic strategies have curative goals with 5-year survival rates of up to 66% across all age groups and anatomical sites (Johnson et al., 2020; Mandal et al., 2016; Solomon et al., 2018).

HNSCC cases are often categorized based on Human Papilloma Virus (HPV) infection status. HPV-16 subtype infection is mainly responsible for the development of HPV-positive tumors, while environmental and lifestyle factors like tobacco smoking and alcoholism are the

major risk factors for HPV-negative tumors. Studies have shown that patients with HPV-positive tumors have better prognosis and treatment outcomes compared to their HPV-negative counterparts (Fakhry et al., 2008).

Epidemiology of HNSCC. As stated above, HNSCC is the seventh most common cancer worldwide, and a 30% increase in the incidence is projected by 2030 (Globocan, 2020). HPV positive cases are more common in the US and Western Europe while HPV negative cases, usually due to carcinogen consumption (areca nut, betel leaf, slaked lime, and/or tobacco products), are common in Southeast Asia and Australia (Hashibe et al., 2007; Mehanna et al., 2013). Patients with HPV positive status are usually diagnosed in their 50s with a median age of 53-55 years, while HPV negative cases are often diagnosed in patients in their 60s with a median age of 66 years. Men have a 2-4 fold higher risk of developing HNSCC than their female counterparts (Johnson et al., 2020). Although the survival for HNSCC has improved in recent years, it is noteworthy that the suicide rates amongst HNSCC survivors are about 3-fold higher than that of other cancers, and second-highest after pancreatic cancer (Osazuwa-Peters et al., 2018).

Biomarkers for HNSCC. Several cellular biomarkers have been studied in relation to HNSCC, especially for HNSCC cancer stem cells. Some of these include CD44, CD133, ALDH1, OCT3, OCT4, SOX2, and NANOG. Within this group, CD44, CD133, and ALDH1 are the most studied and are proposed to significantly impact disease outlook (Johnson et al., 2020). CD44 is involved in intercellular interactions and migration, which may induce metastasis and poor prognosis (A. Faber et al., 2011). Similarly, CD133 is linked to invasiveness and metastasis (Zhang et al., 2010). ALDH1 may also impact HNSCC via cell renewal, invasiveness, and metastasis (Anne Faber et al., 2011; Johnson et al., 2020).

Genomic Landscape of HNSCC. Like other cancers, HNSCC is rife with genomic complexities that include chromosomal changes, DNA promoter methylation, copy number variation, and somatic mutations. Some genomic abnormalities are unique to the HPV-negative tumors while others are common to both categories. For instance, HPV-negative tumors have amplification of genes that express EGFR, REL, BCL6, PI3KCA, TP63, CCDN1, and MDM2 while down-regulation of ATM, CDKN2A, RB1, NOTCH1, and NF1 (Seiwert et al., 2015). Meanwhile, amplification of SOX2, TP63, and PI3KCA are common to both categories of HNSCC. Conversely, HPV-positive tumors have recurrent deletions of TNF receptor-associated factor 3 (TRAF3), amplification of E2F1, and mutation of CDNK2A (Lawrence et al., 2015). Genomic studies have revealed a close relationship in the etiology of HPV-negative HNSCC and lung squamous cell carcinoma (Hammerman et al., 2012). Genome-wide studies have also confirmed the frequent occurrence of TP53, CDKN2A, PTEN, PI3KCA, and NOTCH pathway mutations in HNSCC (Lawrence et al., 2015). However, there is still a lack of actionable targets for HNSCC when compared with melanoma and lung adenocarcinoma. With more recent conflicting reports, the notion that the mutation rates in HPV-negative tumors are higher than HPV-positive HNSCC is still contested (Lawrence et al., 2015; Seiwert et al., 2015).

TP53 and CDKN2A mutations are the most common somatic mutations in HNSCC. TP53 loss of function mutation is more common in HPV-negative tumors. However, in HPV-positive tumors, E6 viral oncoprotein increases the ubiquitination and proteasomal degradation of p53. Contrariwise, CDKN2A is the gene that encodes p16 which inhibits the effects of cyclin D1, CDK4, and CDK6 on tumor suppressor RB1. Somatic mutations, deletions, and promoter hypermethylation of the CDKN2A gene may disrupt this pathway. More so, data from the TCGA consortium indicates that nearly a third of all HNSCC patients have CCDN1 (gene coding Cyclin

D 1) amplification, which is implicated in poor prognosis and resistance to therapies including EGFR inhibition (Solomon et al., 2018).

With mutations and amplifications in over a third of HNSCC cases, PI3KCA is believed to be the most frequently disrupted oncogene in HNSCC (Jung et al., 2018). FGFR amplification is also common in HPV-negative tumors and like lung SCC, FGFR1 amplification and FGFR1 RNA expression could predict response to FGFR1 inhibitors. Additionally, EGFR expression is often elevated in HPV-negative compared to HPV-positive HNSCC, which could worsen prognosis. This led to the development and approval of cetuximab, an EGFR monoclonal antibody inhibitor, used alongside chemotherapy or radiotherapy (Solomon et al., 2018). Other forms of genomic aberrations include RAS family mutations especially HRAS mutations in about 5% of tumors (Ho et al., 2017) and cMET amplification in 2-13% of HPV-negative HNSCC (Hammerman et al., 2012; Solomon et al., 2018).

Standard of Care for Locoregionally Advanced Disease. Surgical resection is the first option considered for patients with HNSCC provided the tumor is within the limits of resection. If surgery would not be beneficial, concomitant chemo-radiotherapy is the treatment of choice. The common practice is fractionation radiotherapy over 7 weeks with 3 cycles of high-dose cisplatin, but some studies have achieved similar outcomes with 6 weeks of fractionation radiotherapy and 2 cycles of high-dose cisplatin (Ang et al., 2010; Oosting & Haddad, 2019). Other forms of chemoradiotherapy include carboplatin with 5FU infusion (Denis et al., 2004) or cetuximab (Bonner et al., 2010). Elderly patients above 70 years appear to benefit less from concurrent chemoradiation. This could be linked to physiological changes in metabolism and an increase in non-cancer-related deaths in this population (Szturz & Vermorken, 2016). Treatment de-intensification was suggested for patients with HPV related oropharyngeal cancers due to the

observed better treatment outcomes in this sub-population, however, reports show that radiotherapy combined with cetuximab offers lower overall survival benefits when compared with high-dose cisplatin, which remains the gold standard (Gillison et al., 2019; Mehanna et al., 2019; Oosting & Haddad, 2019).

Once the surgery is done for patients with resectable tumors, the patient is assessed for risk of recurrence by checking for involved margins of the resection and extranodal lymph node metastasis. If found, the patient is placed on concomitant chemoradiotherapy to reduce the risk of recurrence (Bernier et al., 2005; Bernier et al., 2004; Cooper et al., 2004). In cases where organ preservation is the goal, induction chemotherapy with Docetaxel, cisplatin, and 5FU is recommended, but the benefit of induction over chemoradiotherapy is still being studied (Geoffrois et al., 2018; Oosting & Haddad, 2019; Winquist et al., 2017).

Standard of Care for Recurrent / Metastatic Disease. In metastatic cases where cure cannot be achieved, patients are managed on a cocktail of cisplatin or carboplatin with 5FU and cetuximab closely followed by maintenance with cetuximab. In a situation where malignancy progresses after platinum-based therapy, anti-PD-1 antibodies have been shown to increase overall survival in such patients, which led to the approval of Nivolumab and Pembrolizumab by the FDA for such cases (Cohen et al., 2019; Ferris et al., 2018). However, several studies are still ongoing, and results from these studies could change the treatment focus (Oosting & Haddad, 2019).

Immunotherapy for HNSCC. Considering the fact that the white blood cell population in peripheral blood of HNSCC patients is generally low and comprises mainly regulatory T cells, this cancer is immunosuppressive (Moskovitz et al., 2018). Also, the larger proportion of tumor-infiltrating lymphocytes (Méndez-García et al.) is composed of regulatory T cells, which suppress immunity. However, studies show that tumors with a high CD8+ TIL population have longer

disease-free survival (Lalami & Awada, 2016; Näsman et al., 2012; Nordfors et al., 2013). More importantly, the TILs present the opportunity for immunotherapy as they express immune checkpoint receptors (ICR), which could be blocked by checkpoint inhibitors. Positive results of checkpoint blockade in patients with tumors refractory to initial platinum-based therapy and other clinical trials led to the approval of Nivolumab and Pembrolizumab as first-line treatment for recurrent or metastatic HNSCC and other solid tumors (Ferris et al., 2016). However, the response to ICR blockade alone is not encouraging. Consequently, several studies are trying to identify new checkpoints, others are exploring combination therapies for multiple checkpoint blockade (Wolchok et al., 2013). Another approach is the combination of ICR blockade with radiotherapy to leverage 'abscopal' responses, where immune system activation lingers after radiation exposure (Sharabi et al., 2015). More so, studies show that chemotherapy could stimulate immune response, which presents an opportunity to combine cytotoxic agents with immunotherapy (Moskovitz et al., 2018; Scharovsky et al., 2009).

Cancer Stem Cells and Tumor Resistance

Most cancers have a heterogenous cell population which makes sensitivity to most therapies unpredictable. The cells that escape initial therapy often drive the recurrence of the tumor. This makes it imperative to understand the diverse nature and sensitivities within a tumor to offer more effective therapy options. The observed heterogeneity within a tumor could either be of genomic or epigenetic origin. Cancer stem cells (CSCs) manipulate epigenetic mechanisms to overexpress drug transporters and optimize their DNA repair ability. Consequently, most cytotoxic drugs will eliminate the majority of cancer cells but are ineffective against CSCs, which can eject cytotoxic drugs via efflux pumps (Lytle et al., 2018). Also, CSCs resist radiotherapy by activating checkpoint kinases 1 and 2, to heighten their DNA repair ability in glioblastomas (Bao et al., 2006)

or by relying on PCNA-associated factor (PAF)-driven translesion DNA synthesis in gliomas (Ong et al., 2017).

Stem Cell Resistance to Targeted Therapies

Recent advances in oncology research have championed the discovery of targeted therapies that specifically inhibit mutations that drive the progression of tumors. The first of this group was imatinib, which effectively targeted the BCR-ABL in chronic myeloid leukemia, but about 50% of the patients relapsed after initial full remission once imatinib was withdrawn (Kimura, 2016). This relapse was largely attributed to CSCs and their ability to activate alternative survival pathways (Chu et al., 2011). Similarly, in lung cancer, EGFR targeted therapies have upregulated stem cells that resort to NOTCH signaling to ensure survival without any new mutation events (Arasada et al., 2014). Furthermore, the poor response to immunotherapy options could be linked to stemness, because in highly undifferentiated tumors, immune infiltration is low which leads to diminished PDL1 signaling (Lytle et al., 2018; Malta et al., 2018).

Tumor Microenvironment and CSC Resistance

Besides the inherent ability of CSCs to evade therapeutic assault, studies show that they may also get some help from the tumor microenvironment especially in solid tumors (Lytle et al., 2018). For instance, endothelial cells support stem cells by secreting nitric oxide which enables NOTCH signaling in gliomas, whereas the hypoxia resulting from endothelial cell inhibition by VEGF blockade can promote stemness in non-stem cells (Charles et al., 2010; Vredenburgh et al., 2007). Apart from endothelial cells, fibroblasts also contribute to the survival and resistance of cancer stem cells by secreting cytokines like IL-6 and IL-8 that promote stem cell survival (Lytle et al., 2018). This relationship has been reported in NSCLC (Chen et al., 2014), basal cell carcinoma (Sneddon et al., 2006), and colorectal cancer (Vermeulen et al., 2010).

Anti-Tumor Agents from Natural Sources

Some of the most impactful discoveries in the history of modern medicine have been found in nature. As early as the eighteenth century, physicians in Europe had discovered an ancient technique called 'variolation'. This technique conferred immunity to smallpox by harnessing the natural defenses of the human body and consequently revolutionized vaccination across Europe (Aboul-Enein et al., 2012). The twentieth century witnessed more discoveries from nature that have defined our modern era. For example, the discovery of penicillin in the fungus *Penicillium notatum* stemmed the carnage from bacterial infections. More so, the discovery of insulin in dogs was phenomenal in our understanding of diabetes and other endocrine disorders.

However, it is noteworthy that before these record discoveries, man has always obtained herbs from his immediate environment to alleviate symptoms and cure diseases with varied success. Over time, this health-seeking behavior has led to the discovery of secondary metabolites like alkaloids, glycosides, and tannins that have been refined and developed for different indications in modern medicine including oncology (Seca & Pinto, 2018).

Today, as our understanding of cancerous diseases becomes clearer, scientists still seek solutions to this group of diseases that have devastated humanity, and nature as an invaluable resource is not left out of the search.

Microbes

Microorganisms are a prolific and reliable source of medicinal agents. Since the discovery of Penicillin from *Penicillium notatum,* science has further explored microorganisms as a potential source of therapeutic agents. Consequently, many antibiotics have been developed from the secondary metabolites of microorganisms. Microbes are regarded as the most important natural source of anti-tumor agents due to their wide distribution and diversity (Khazir et al., 2014). The

popular anti-tumor antibiotics obtained from microbial sources include members of the anthracycline, bleomycin, actinomycin, mitomycin, and aureolic acid families. The majority of the clinically useful anticancer antibiotics were isolated from Streptomyces species, some of which includes daunorubicin, doxorubicin, idarubicin, epirubicin, bleomycin A and B, peptolides (actinomycin D), mitosanes (mitomycin-C), and glycosylated anthracyclines (mithramycin) (Cragg & Newman, 2000). New anti-tumor antibiotics were also approved in recent years. For example, a derivative of sirolimus isolated from Streptomyces species everolimus (RAD-001), which acts by inhibiting mTOR was approved by the FDA for the management of pancreatic neuroendocrine tumors. Its analogs have had subsequent approvals for different cancer indications in recent years (Khazir et al., 2014).

Actinomycins. The term actinomycin was coined by Waksman and Woodruff in 1940. This was a tentative name for the active substances they isolated from a species of soil Actinomyces. At the time, their studies focused on elucidating the bacteriostatic and bactericidal properties of two active substances, which they designated Actinomycin A and Actinomycin B (S. A. Waksman & H. B. Woodruff, 1940). By the 1960s, actinomycins were considered as some of the most potent antitumor agents known and were actively indicated for the treatment of different neoplasms, but their clinical application was limited by toxicity (Reich, 1963; Reich & Goldberg, 1964). It was then demonstrated that their antitumor activity was exerted by inhibiting DNA-dependent RNA synthesis (Goldberg & Rabinowitz, 1962), which was only overcome by increasing concentration of DNA (Hurwitz et al., 1962; Reich, 1963; Reich et al., 1962). This effect leads to inhibition of protein synthesis and eventually, partial inhibition of DNA synthesis. However, actinomycins are primarily toxic to those cellular activities directly involving DNA, because actinomycins form complexes with DNA and not RNA.

By far the most studied and clinically applied actinomycin is actinomycin D, which has been used in the treatment of various tumors since the 1950s. Actinomycin D inhibits both RNA and DNA synthesis, with the former more markedly affected (Hurwitz et al., 1962). Besides, the presence of a guanine base is required for the phenoxazone ring on the actinomycin D molecule to form a complex with DNA (Reich & Goldberg, 1964). Figure 1 shows the chemical structure of actinomycin D with the phenoxazone ring flanked by two cyclic pentapeptide lactone rings.

Although newer and highly effective chemotherapeutics have been discovered over the years, actinomycin D still has clinical application in combination therapy with newer agents in efforts to overcome resistance.

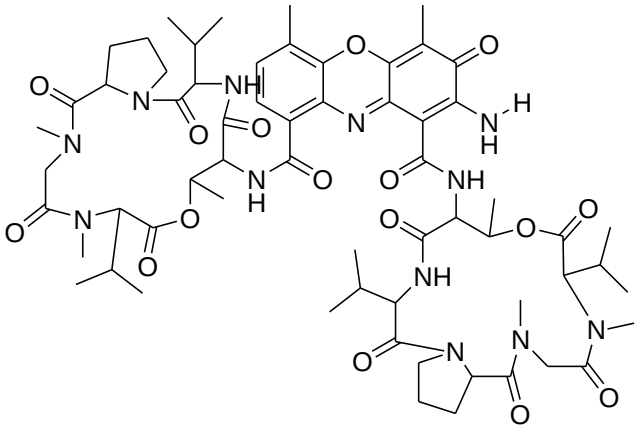


Figure 1: Chemical Structure of Actinomycin D

Clinical Applications of Actinomycin D. Actinomycin D (ACTD) is indicated in the treatment of several malignancies such as Wilms tumor, Ewing's sarcoma, childhood rhabdomyosarcoma, gestational trophoblastic leukemia to mention a few. In the management of gestational trophoblastic leukemia, Pulsed IV actinomycin D (1.25 mg/m2 to a maximum 2 mg single dose), repeated every 14 days or a 5-day actinomycin D (0.5 mg IV), repeated every 14 days are the most common regimens. Studies comparing methotrexate and Actinomycin D for this

indication showed that Actinomycin D is more likely to achieve primary cure and less likely to fail as first-line treatment (Biscaro et al., 2015; Lawrie et al., 2016).

In the treatment of rhabdomyosarcoma, based on the risk level of the patient, Actinomycin D is used in combination with other agents like; vincristine and cyclophosphamide but the efficacy and toxicity of the current regimen are not encouraging (Hosoi, 2016).

While surgical resection and radiotherapy remained the first-line option for patients with Ewing's sarcoma, in the 1970s Vincristine, Actinomycin D, and cyclophosphamide (Hammerman et al.) regimens were used as adjuvant therapy to control metastasis and as neoadjuvant therapy to enhance local control. However, newer combinations like Vincristine, Doxorubicin, and Cyclophosphamide (VDC) have actinomycin replaced with doxorubicin in current therapy regimens for Ewing's sarcoma (Gaspar et al., 2015).

In the management of Wilms' tumor, about 67% of patients are treated with vincristine and actinomycin D as preoperative or adjuvant chemotherapy depending on the context (Spreafico & Bellani, 2006). Also, early studies of patients with Wilms' tumor indicate that the administration of actinomycin D at the point of surgery or local radiotherapy may prevent metastasis (Farber et al., 1960).

However, due to lack of specificity of action, actinomycin D is a severely toxic drug with extra-tumoral effects on tissues with high growth rates like the bone marrow, liver, and gastro-intestinal epithelium (Philips et al., 1960). There is also a risk of necrosis at injection sites due to extravasation (Farber et al., 1960). These toxic effects limit the clinical application of actinomycin D.

In the context of aerodigestive tract tumors, especially lung cancer, actinomycin D was studied clinically in combination with vincristine because in vitro studies had shown that

vincristine improved the therapeutic effects of actinomycin D. Also, the unique toxicity profile of both drugs, which was non-additive made the combination attractive. The results of the study showed that the combination had impressive antitumor activity in patients with lung cancer irrespective of cell type (Chanes et al., 1971).

p53 as a Tumor Suppressor

p53, commonly referred to as the ‘‘guardian of the genome’’ is a protein that plays an important role as a tumor suppressor within the cell. It was initially classed as an oncogene but further investigation revealed that it suppressed tumor growth and oncogenic activation (Finlay et al., 1989). Today, it is arguably the most important tumor suppressor and the most frequently mutated/ altered gene in solid tumors. p53 is encoded by the TP53 gene and regulated via negative feedback mechanisms by MDM2 and its homolog MDMX. The tumor suppressor protein is activated by cellular stress signals such as oxidative stress, DNA damage, oncogenic stress, hypoxia, etc. (See Fig. 2) (Joerger & Fersht, 2016). As a transcription factor, activation of p53 upregulates the expression of downstream target genes. Consequently, these target genes play important roles in cell cycle arrest (p21), DNA repair (Mgmt), senescence (Pai1), anti-angiogenesis (Tsp1), apoptosis (PUMA), etc. (Joerger & Fersht, 2016).

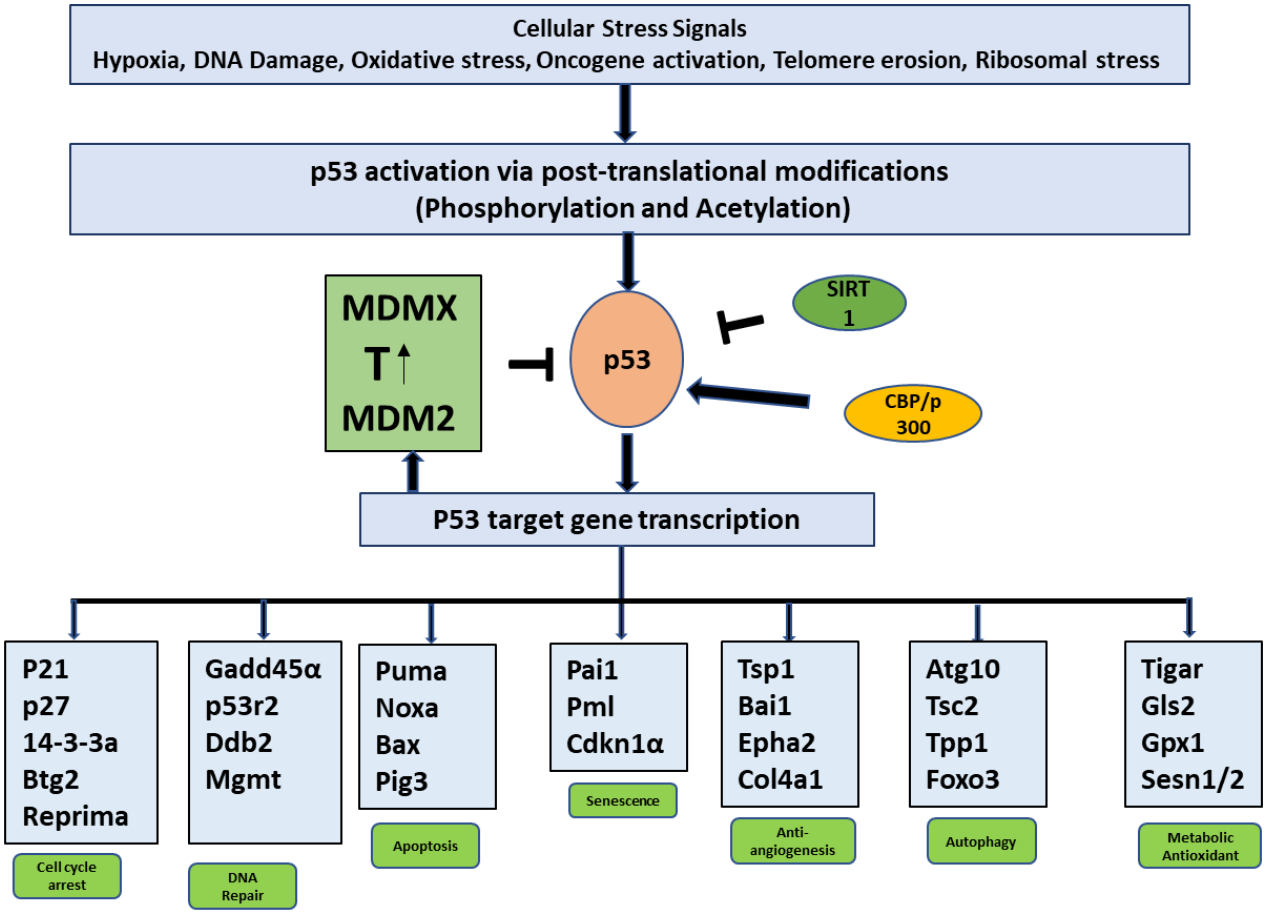


Figure 2: p53 pathway illustration.

The p53 pathway is activated by cellular stress signals that lead to post-translational modifications and stabilization of the protein. Upon activation, p53 transcribes target genes that are responsible for its tumor suppressor activities like, cell cycle arrest, DNA repair, apoptosis, senescence, etc. The cellular level of p53 is tightly regulated by a negative feedback mechanism of its target gene Mdm2/Mdmx. The illustration was adapted with modifications from (Joerger & Fersht, 2016)

While p53 protein is considered an important tumor suppressor, it is often inactivated in most tumors either by mutation of the TP53 gene or pathway deregulation. p53 is inactivated by mutation in more than 50% of human tumors and the prevalence of mutations varies with cancer type and developmental stage- from as low as 5% and 10% in cervical cancer and leukemia, to as high as 80% and 90% in small cell lung cancer and ovarian cancer, respectively (Joerger & Fersht, 2016; Kastenhuber & Lowe, 2017; Leroy et al., 2014).

To guard the genome, the tumor suppressor p53 could either halt the cycle of cells with DNA damage, which prevents the accumulation of oncogenic alterations. Alternatively, it could prevent the proliferation of cells with DNA damage to prevent the consequences of oncogenic alterations in subsequent generations. Either way, a loss of p53 function will allow oncogene-expressing cells to thrive without check, which will ultimately result in malignancy (Kastenhuber & Lowe, 2017; Livingstone et al., 1992; Serrano et al., 1997).

p53 is known to have several distinct biological effects, which may be due to stimulus-dependent posttranslational modifications (PTMs) of p53 that lead to increased affinity for specific target genes. For example, phosphorylation of the tumor suppressor at the Ser 46 site activates its proapoptotic effects, whereas Protein arginine methyltransferase (PRMT)-methylated p53 will activate p21 and cell cycle arrest more readily (Kastenhuber & Lowe, 2017; Kumari et al., 2014).

More so, the manner of expression (steady or pulsed signaling) could determine target gene bias. While the p21 promoter is sensitive to short impulses of p53 expression and activity, the proapoptotic FAS favors a steady activation to induce apoptosis (Espinosa et al., 2003; Kastenhuber & Lowe, 2017; Morachis et al., 2010).

While it is considered a fact that p53 has tumor-suppressive abilities, the effector functions of the protein that are responsible for these abilities are still disputed. Many agree that apoptosis

and senescence are vital to the tumor suppressor role of p53, but recent studies show that they might be dispensable. Valente and colleagues demonstrated how mice deficient in downstream effectors of p53 (p21, PUMA, and FOXO) did not develop thymic lymphoma (Valente et al., 2013). However, it is known that multiple effectors are responsible for target effects of p53, and like other downstream effects of p53, the cell cycle arrest effect is not entirely dependent on the presence of p21.

There is a notion that p53 effects are context-specific. Consequently, some studies tried to revive the p53 of established tumors in mice and observed varied effects. In some cases, reactivation of p53 induced massive apoptosis while in others it triggered cell differentiation and loss of cell renewal (Kastenhuber & Lowe, 2017; Messina et al., 2012).

As mentioned above, TP53 mutation is the most common mutation in human tumors (Olivier et al., 2010). They can be classified into contact and conformational mutants. The contact mutants (e.g R273H) lose their ability to make necessary contacts required for DNA binding, while the conformational mutants (R175H) are slightly unfolded which leads to loss of the zinc and DNA binding ability (Pfister & Prives, 2017).

In many cases, this results in effects beyond the loss of function of p53. In most cases, loss of function is associated with p53 mutants, but studies show some mutants retain selective functions while others have entirely new (neomorphic) functions in some cases aiding metastasis and invasion (Freed-Pastor & Prives, 2012; Muller et al., 2013; Shirole et al., 2017). It is believed that the gain-of-function of mutant p53 is due to interactions with other transcription factors e.g p63, p73, E2F1, MED1, etc. (Pfister & Prives, 2017).

Some therapeutic approaches have been developed to circumvent the inactivation of p53 in many cancers. One approach is to use MDM2 inhibitors to increase the stability of p53 in cells

with wild-type p53, an example is the use of Nutlins (Vassilev et al., 2004). Another approach is to use agents that will restore the wild-type function of mutant p53 either by stabilizing the DNA binding domain or maintaining a more stable conformation of the protein. Some of these agents are currently assessed in clinical and preclinical studies (Cheok & Lane, 2017; Deneberg et al., 2016). Also, mutant p53 tumor mechanisms of invasion and metastasis could be destabilized by inhibiting EGFR and PDGFR pathways (Aschauer & Muller, 2016; Kastenhuber & Lowe, 2017; Weissmueller et al., 2014). Yet another way is to use mutant p53 proteins as neoantigens that can trigger a p53-specific immune response for the development of vaccines (Roth et al., 1996).

Apoptosis

Apoptosis, often referred to as programmed cell death, is an essential evolutionarily conserved tumor suppressor function of p53 (Yu & Zhang, 2005). It is often viewed as a way of eliminating cells with DNA damage or growth abnormalities to prevent the development of malignant clones. It completes the continuum of growth arrest and DNA repair strategies to preserve genetic integrity (Bellamy, 1997). There are two main pathways of apoptosis observed in mammalian cells, the extrinsic and intrinsic pathways.

The extrinsic pathway proceeds by binding of TNF family protein TRAIL to its receptors DR4 and DR5. This leads to some structural changes of the receptor's intracellular death domain and the formation of a death-inducing signaling complex (DISC). Subsequently, FADD is recruited and activation of caspase 8 and 10 results in cleavage of caspase 3, and ultimately cleavage of death substrates ensues. Contrariwise, the intrinsic pathway is stimulated by cellular stress signals such as hypoxia, DNA damage, and cell cycle checkpoint defects to mention a few. Consequently, proapoptotic Bcl2 proteins (PUMA, NOXA, Bax, and Bak) are activated, which ensure mitochondrial membrane compromise and release of cytochrome c and SMAC/DIABLO

into the cytosol. Cytochrome c by binding APAF-1 forms apoptosome, which activates caspase 9. Caspase 9 ultimately activates caspase 3, 6, and 7 which execute apoptosis (Liu et al., 2017; Wang & El-Deiry, 2003).

As displayed in Figure 3, both pathways are not entirely isolated, there is some crosstalk observed. For instance, caspase 8 cleaves Bid, and truncated Bid is translocated to the mitochondria to interact with Bax and Bak to ensure the release of cytochrome c to the cytosol. This presents a possible link between both pathways of apoptosis (Green, 2000; Liu et al., 2017; Wang & El-Deiry, 2003).

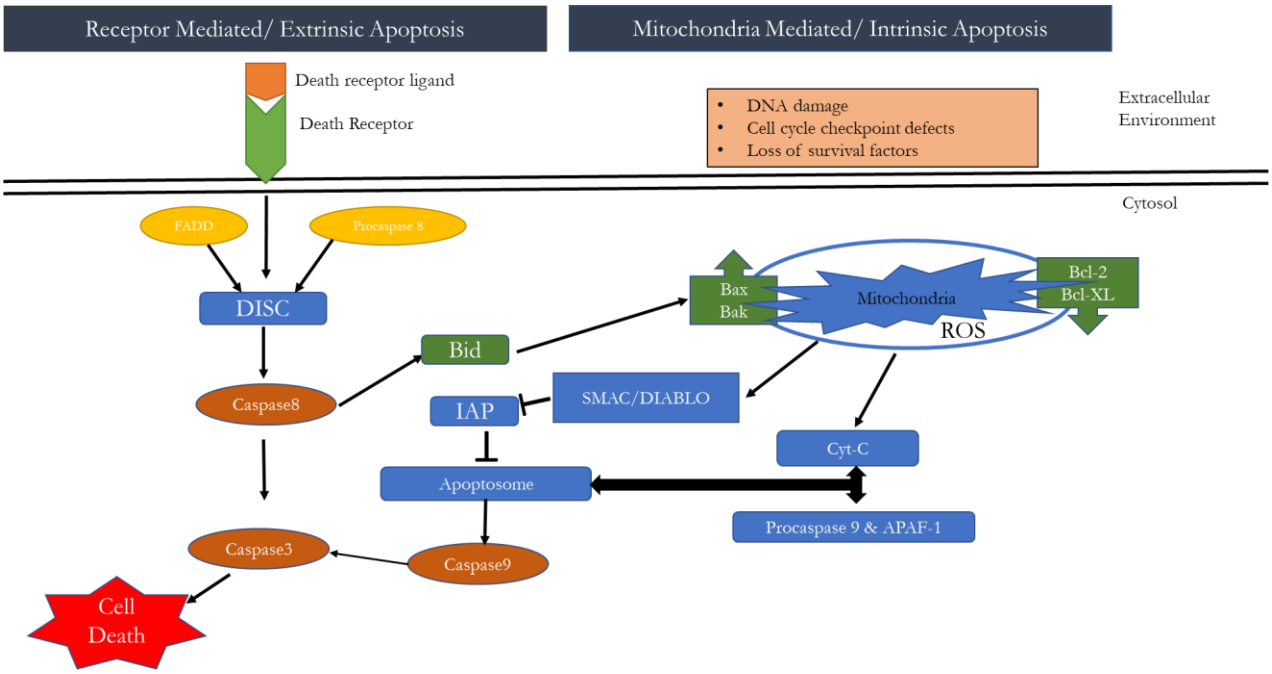


Figure 3: Apoptotic pathways.

The extrinsic pathway is a direct pathway that proceeds with the extracellular activation of death receptors, which leads to the formation of a death-inducing signaling complex (DISC). This triggers the activator caspase (Caspase 8) and effector caspase (Caspase 3). On the other hand, the intrinsic pathway is activated from within the cell often due to cellular stress signals. It is regulated by a delicate balance of pro and anti-apoptotic Bcl-2 proteins, which could lead to the release of

apoptotic mediators, formation of an apoptosome complex, and ultimately the activation of effector caspase (Caspase 3). Adapted with modifications from (Wang & El-Deiry, 2003).

Transcription- Dependent Apoptosis

p53-dependent apoptosis is triggered by DNA damage, oncogenic activation, hypoxia, heat shock, and certain cytokines or cytokine deprivation. Also, death receptor (Fas, DR4, DR5) activation is not required for p53 dependent apoptosis, but it is known that through activation of these receptors, p53 could trigger or enhance apoptosis induced by TRAIL and chemotherapeutics (Liu et al., 2004; Vousden & Lane, 2007). Some studies also report DR4 and DR5 as downstream transcriptional targets of p53 (Liu et al., 2004; Sheikh et al., 1998). However, it is often suggested that the intrinsic pathway is primarily utilized for p53 dependent apoptosis, with the extrinsic pathway playing a complementary role (See Fig. 4) (Fridman & Lowe, 2003). By far, DNA damage is the most studied stimulant of p53 dependent apoptosis. By binding to DNA strand breaks, p53 is stabilized and activated. It is suggested that activation rather than an accumulation of the protein is necessary for engaging downstream effectors (Bellamy, 1997).

The loss of p53 dependent apoptosis accelerated tumor development in many cell and animal studies (Parant & Lozano, 2003). As a transcription factor, p53 binds to DNA in a sequence-specific manner that leads to activation of some downstream effectors of several cellular functions including apoptosis. However, tumors with nonfunctional p53 lack this ability and are resistant to the p53 mediated apoptosis resulting from DNA damage or oncogene activation (Yu & Zhang, 2005). It is notable, however, that some p53 mutants have retained certain functions of wild type p53 that could play a role in tumor suppression and chemotherapy response (Timofeev et al., 2019).

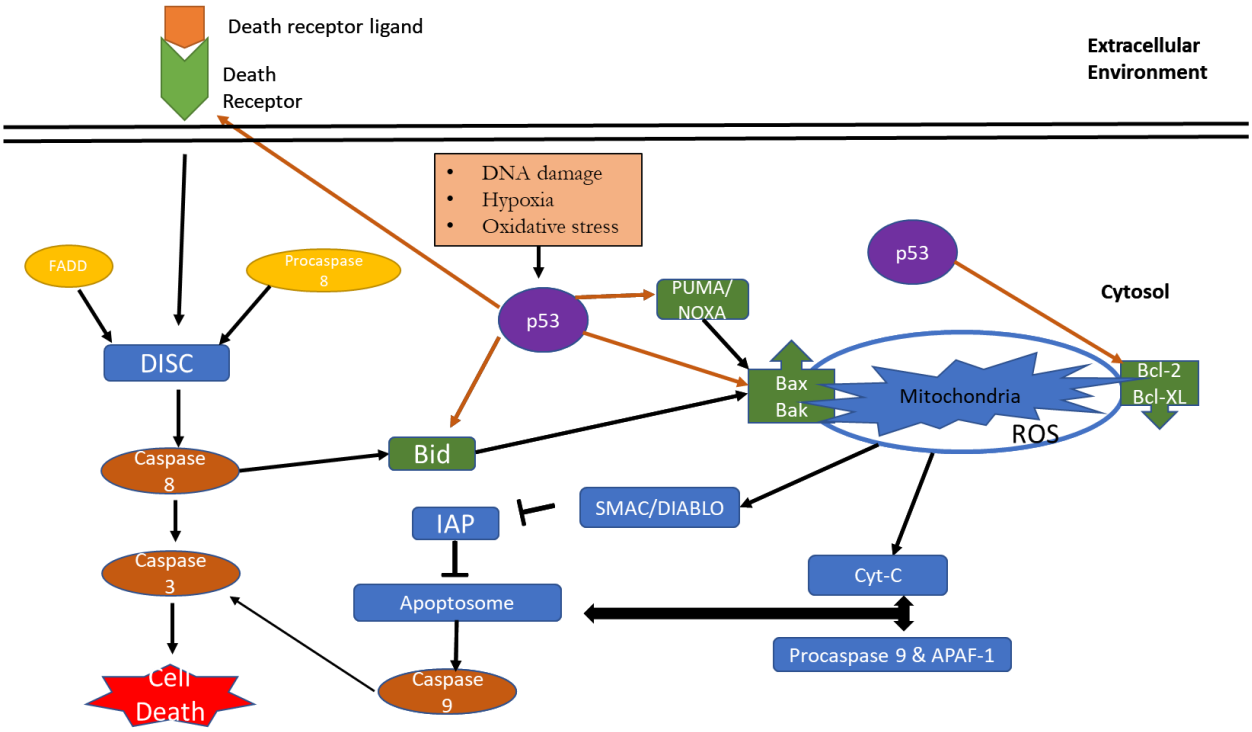


Figure 4: p53-dependent apoptosis.

p53 is stabilized and activated by cellular stress signals like DNA damage, hypoxia, and oxidative stress. Activated p53 (i) activates pro-apoptotic Bcl-2 such as BAX, BAK, PUMA, NOXA; (ii) activates death receptors (DR4, DR5) to trigger extrinsic apoptosis; (iii) translocates to mitochondria to trigger transcription-independent apoptosis. Adapted with modifications from (Schuler & Green, 2001; Wang & El-Deiry, 2003).

Transcription-Independent Apoptosis

Since no single target gene can fully explain the apoptotic effects of p53, this suggests that p53 induces apoptosis via several mechanisms, which include transcription-independent mechanisms. Contrary to earlier suggestions that p53 accumulation was not critical to its apoptotic effects, p53 accumulation in the cytosol and mitochondria has been argued to be essential to the transcription-independent apoptotic effects of p53 (Ho et al., 2019).

As displayed in Figure 4, p53 can directly activate Bcl2 proteins (Bax and/or Bak) on the mitochondria in favor of mitochondrial outer membrane permeabilization (MOMP) and ultimately apoptosis induction (Speidel, 2010). Also, p53 can increase the release of calcium by the endoplasmic reticulum, which will cause calcium overload and a compromise of the mitochondrial morphology leading to apoptosis (Liu et al., 2017). It is notable that some contact and structural mutants of p53 still possess the ability to activate Bax in vitro and in vivo but did not exhibit apoptosis under in vivo conditions. However, a clear understanding of the retention of this proapoptotic activity may be critical to treating patients with p53 mutation (Castrogiovanni et al., 2018; Pietsch et al., 2008; Speidel, 2010). Another non-transcriptional apoptotic effect of p53 is its binding to anti-apoptotic Bcl-2s (Bcl-XL and Bcl-2). This interaction supposedly liberates Bax or Bak from the inhibition of antiapoptotic Bcl-2 proteins. Others have also demonstrated that the interaction between p53 and the antiapoptotic Bcl-XL/Bcl-2 hinders p53's proapoptotic functions. This gives room for further research to validate both proposals (Chipuk et al., 2005; Deng et al., 2006; Speidel, 2010).

p53 Independent Apoptosis

The fact that p53 is mutated in most tumors and the observation that p53-mutated cells still undergo apoptosis lends credence to the suggestion that p53 independent mechanisms of apoptosis occur via some backup systems. One of such backup strategies is the activation of p53 homologs p63 and p73. Some studies have shown that following DNA damage, p73 could be activated by E2F1 and exhibit proapoptotic activity without p53. The downstream effects of p73 were observed to be exerted by transactivation of PUMA and NOXA, which led to Bax-induced MOMP (Ray et al., 2011). Another system is the activation of other families of tumor suppressor proteins, for example, the FoxOs. As transcription factors, FoxOs upregulate the expression of pro-apoptotic

Bcl-2 proteins that facilitate mitochondrial permeabilization. They also enhance the extrinsic pathway of apoptosis by upregulating death receptor ligands like TNF-related apoptosis-inducing ligand (TRAIL) (Zhang et al., 2011).

The extrinsic apoptotic pathway is somewhat independent of p53 in inducing apoptosis. In some cells (Type 1 cells), the activation of caspase 8 by DIABLO is enough to activate apoptosis effector caspases 3,6, and 7 without the involvement of the intrinsic arm of the apoptotic pathways. Whereas in other cells (Type 2 cells) activation of caspase 8 is not sufficient to induce apoptosis, it requires amplification of the apoptotic signal by the intrinsic pathway through truncated BH3 protein, tBid (Gonzalvez & Ashkenazi, 2010; Wang & El-Deiry, 2003). Consequently, the latter may not be considered entirely independent of p53 interference.

Oxidative stress-induced apoptosis is another mechanism of apoptosis that may be independent of p53 interference. Mendez-Garcia and colleagues studied how suppression of Nrf2 (nuclear factor erythroid 2-related factor 2), a regulator of antioxidant genes with cytoprotective functions, would affect oxidative stress-induced apoptosis by curcumin in p53 deficient cells. In their studies, they observed downregulation of Nfr2 target genes (HMOX1, GCLC, GCLM, and TXN) in the absence of p53 activation, which suggests that Nfr2 inactivation and oxidant-induced apoptosis could occur independently of p53 (Méndez-García et al., 2019).

In addition, it had been shown that reactive oxygen species can cause NF-κB induced transcription of FAS ligand, which promotes apoptosis in Jurkat cells (Bauer et al., 1998). Burger and coworkers also explored p53-independent apoptotic mechanisms in testicular germ cell tumor cell lines. These cells had differences in p53 expression and functionality and were exposed to various apoptotic stimuli (cisplatin, doxorubicin, gamma irradiation, and cell-permeable C2 ceramide). Their findings identified distinct apoptotic pathways (including a Fas-mediated

activation of apoptosis) independent of p53 status in testicular tumor germ cell lines (Burger et al., 1999).

Resistance to Apoptosis

A delicate balance must be maintained between cell proliferation and cell death to ensure tissue homeostasis. When the former outweighs the latter, this balance is breached, and this may give room for carcinogenesis. More so, the cell death arm is controlled by cellular processes like apoptosis, autophagy, and necrosis. Many cancer cells have developed mechanisms to resist apoptosis, which ensures tumor progression and resistance to therapy because most therapeutic options available (chemotherapy, radiotherapy, and immunotherapy) are geared towards activating apoptosis (Fulda, 2009).

There are several ways cancer cells evade apoptotic signals. One is the deregulation of death receptor expression and pathway functions. For instance, studies have reported decreased expression of CD95 in leukemia and neuroblastoma which led to chemotherapeutic resistance (Friesen et al., 1997; Fulda et al., 1998). Also, genetic aberrations in some cancers including HNC, and lung cancer have led to the loss of expression of TRAIL receptors responsible for inducing apoptosis (Fisher et al., 2001; Lee et al., 1999). Other mechanisms involving the death receptor pathways include aberrant expression of decoy receptors (Fisher et al., 2001) and inhibition of caspase 8 by mutation or epigenetic changes (Hopkins-Donaldson et al., 2003; Mandruzzato et al., 1997).

Another major mechanism of apoptosis evasion is through deregulation of the mitochondrial pathway. At the core of this pathway is the Bcl-2 family of proteins which consist of pro (Bax and Bak) and anti-apoptotic (Bcl-2, Bcl-XL, and Mcl-1) members. Consequently, the sensitivity to apoptosis is determined by the ratio of expression of anti-apoptotic to pro-apoptotic

Bcl-2 proteins (Fulda, 2009). Certain events like overexpression of anti-apoptotic Bcl-2 proteins, inactivation of Bax and BH3-only proteins by mutations, and epigenetic silencing may be responsible for apoptotic resistance of tumor cells (Fulda, 2009).

Bioassays For Growth Inhibition and Apoptosis Evaluation

Sulforhodamine (SRB) Assay for Growth Inhibition

This technique was developed by Skehan and coworkers in 1990 as a new method to determine the cytotoxic and growth inhibitory effects of anticancer agents (Skehan et al., 1990). This technique depends on SRB's ability to bind to cellular proteins under mildly acidic conditions and its extraction and solubilization in the presence of a base. The optical density of the solubilized dye can be used as a linear measure of cell protein mass, cell number, and cell density (Orellana & Kasinski, 2016). It is particularly remarkable for its rapidity and cost-effectiveness when conducting numerous cellular studies. More so, it gives a reproducible and stable end point that is time-insensitive, which is an advantage over tetrazolium assays (Voigt, 2005). In the determination of 50% inhibitory concentration (IC_{50}), which is the drug concentration that yields half of the cells compared to the control group, the SRB assay results were comparable with tetrazolium assays or clonogenic assays (Griffon et al., 1995; Rubinstein et al., 1990). Subsequently, Keepers and colleagues reported better linearity and sensitivity with the SRB assay which was not dependent on the cell lines tested (Keepers et al., 1991). Figure 5 shows the chemical structure of sulphorhodamine B.

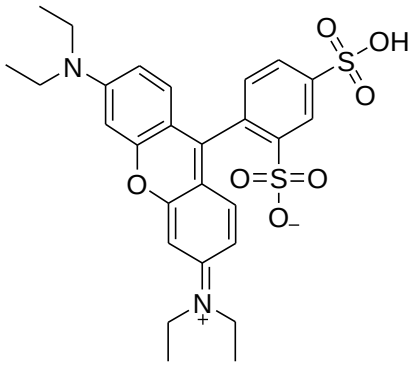


Figure 5: Chemical Structure of Sulforhodamine B

Apoptotic Assays

When cells undergo apoptosis there are two stages involved, the early and the late stage. In the early stage, the cell membrane becomes compromised and this leads to the translocation of phosphatidylserine (PS) from the inner layer of the cell membrane to the external surface. This phase of apoptosis can be detected and quantified by Annexin V staining. This technique is based on Annexin V's ability to bind strongly to phosphatidylserine in a Ca^{2+} dependent manner.

However, necrotic cells also expose phosphatidylserine, but the difference lies in the timing of exposure. Necrotic cells expose PS instantly while for apoptotic cells, PS exposure is not instant but gradual. There have been cases of Annexin V binding to normal cells without any indication of cell death, also some have reported cells that won't bind annexin V, living or dead (Crowley et al., 2016). To avoid false positives, a dye exclusion test with nuclear stains such as PI or 7-AAD should be conducted along with annexin V staining to confirm membrane integrity in early apoptosis and nuclear staining in late apoptosis (Crowley et al., 2016; Vermes et al., 1995).

In late apoptosis, cell membrane integrity is compromised. Nuclear stains like propidium iodide (PI) and 7-AAD can easily penetrate the membrane to detect apoptotic cells. These stains would not detect live or early apoptotic cells because of their intact cell membrane. This makes them suitable to distinguish between early and late apoptotic cells. However, PI is the more widely

used nuclear stain because of its stability, cost-effectiveness, and high specificity for dead cells (Rieger et al., 2011). A disadvantage is the lack of verified concentration for apoptosis quantification. Zembruski and colleagues proposed 5, 10, and 20ug/ml of 7-AAD (Zembruski et al., 2012).

Biochemical Assays (SDS-PAGE & Western Blotting)

Since the 1970s, many researchers have used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) together with the western blot technique to study the molecular mechanisms of drugs. This method was devised by Towbin and modified by Burnette to the more common form used today (Burnette, 1981; Towbin et al., 1979). The technique is an offshoot of the northern and southern blots for RNA and DNA detection, respectively. It involves the separation of denatured proteins from a cellular or tissue extract by gel electrophoresis. This separation is achieved by protein-specific characteristics like molecular weight, charge, and/or isoelectric point that determine the speed and direction of migration through the gel when voltage is applied. Due to electric charge diversity within a protein sample, SDS, an anionic detergent is added to confer a uniform negative charge to all the proteins in the sample. This enables unidirectional migration of the individual proteins when voltage is applied (Jensen, 2012).

The gel electrophoresis is closely followed by the protein transfer or blotting step, which involves the transfer of proteins from the gel to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Nitrocellulose is the more commonly used but PVDF has a higher protein binding capacity and durability. There have been several modifications to the blotting method since it was first introduced. Common methods include simple diffusion, vacuum-assisted solvent flow, and electrophoretic elution (electroblotting). However, electroblotting is by far the most preferred because of its completeness and rapidity. Electrophoretic elution could either be a wet

process that involves complete immersion of the gel-membrane sandwich in transfer buffer, or a semi-dry method where the gel-membrane assembly is placed between absorbent paper inundated with transfer buffer (Kurien & Scofield, 2006).

Gene Expression Analysis -Quantitative Polymerase Chain Reaction (qPCR)

The qPCR technique was introduced by Mullis and colleagues in the 1980s when they used it to amplify a DNA sequence of the human beta-globin gene (Mullis & Faloona, 1987; Saiki et al., 1985). Since then, the technique has been adopted widely by molecular biologists because it is an easy, rapid, and effective way to monitor gene expression. The method is based on the ability of polymerase enzyme to amplify short strands of DNA or RNA in the presence of short terminal oligonucleotides (primers) all within a sample. In principle, the amplification consists of several cycles of denaturation and renaturation of the target nucleic acid. However, the progression of the reaction is temperature-specific. Denaturation of DNA occurs at about 95℃ while annealing of primers to denatured DNA is optimal between 55 and 72℃. For the extension of the annealed DNA, the reaction temperature is raised to 72℃ because of the polymerase enzyme activity, hence the extension rate is high between 70-80℃ (Ramesh et al., 1992). In cases where the starting material is RNA, an extra step catalyzed by reverse transcriptase enzyme, which converts the target RNA to cDNA, is required and often achieved before the typical PCR. But Myers and Gelfand demonstrated the dual activity of Thermus thermophilus as an RNA reverse transcriptase and DNA polymerase, which permits both steps to be completed by one enzyme in the same reaction tube (Myers & Gelfand, 1991; Ramesh et al., 1992).

Significance of the Project

Aerodigestive tract cancers including lung, HNC, and esophageal cancers form a major group of tumors that raise a public health concern. Lung cancer is the most common and deadliest

of all malignancies worldwide. While several new therapies have been developed and clinically applied for lung cancer, the 5-year survival is still about 20%. Moreover, resistance often ensues due to genomic alterations. On the other hand, HNC is the seventh most common cancer worldwide. Like lung cancer, HNC is also subject to genomic alterations for which newer therapeutic targets have gained little success (Cramer et al., 2019).

Furthermore, most cytotoxic chemotherapy agents trigger p53 dependent apoptosis by activating genotoxic, metabolic, or oxidative stress signals. However, besides their toxicity towards normal cells, p53 is mutated in half of all human tumors making therapy with these agents ineffective. But some studies have identified retention of some wild type p53 activities in p53 mutated tumors. This may stimulate sensitivity to conventional chemotherapy drugs like Doxorubicin whose toxicity involves ROS and DNA damage dependent mechanisms (Timofeev et al., 2019). There are reports of combination strategies that may lower the effective dose of cytotoxic agents and may eliminate the terrible adverse effects of these agents (Green et al., 2019; Guo et al., 2012; Yin et al., 2019). Low dose Actinomycin D in combination with gemcitabine was reported to cause reversible cytostatic effects on normal keratinocytes with wild type p53. This protected normal cells but not p53 mutant or deficient tumor cells from the cytotoxic effects of gemcitabine. This approach is known as p53-based cyclotherapy, and it is being employed to spare normal cells with wild-type p53 from the cytotoxic effects of systemic chemotherapy (Rao et al., 2013; van Leeuwen et al., 2012).

In aerodigestive cancers, some studies have shown that actinomycin D can sensitize NSCLC to TRAIL-induced apoptosis, which may be linked to p53 status (Guo et al., 2012). Others have attributed actinomycin D -induced apoptosis in small cell lung cancer to suppression of Mcl-1 and upregulation of NOXA (Xu & Krystal, 2010). Moreover, reports show that combining

actinomycin D with telmisartan can improve tumor permeability and targeting of cancer stem cells, which may be beneficial in resistant cases (Green et al., 2019). In addition, low-dose actinomycin D is gaining traction as an alternative to nutlin-3 (an MDM2 inhibitor) in p53- based cyclotherapy approaches (Choong et al., 2009).

Nevertheless, while actinomycin D is an established and widely studied antitumor agent, which has been used clinically for the management of pediatric tumors, its activity in aerodigestive cancers has not been fully explored. This presents an opportunity to elucidate the activity of actinomycin D in aerodigestive tract tumors. Defining its mechanism will pave the way for a more comprehensive therapeutic approach to manage aerodigestive tract tumors.

CHAPTER 3

MATERIALS & METHODS

Cell Culture

Cells and Reagents

HNSCC cell lines used in the present study have been previously described (Zhao et al., 2011). MDA686TU (Tu 686), a primary tongue cancer cell line, was procured from Dr. Peter G. Sacks (New York University College of Dentistry, New York, NY, USA) in 2014. JHU022, UM-SCC47, 93-VU-147T were procured from Dr. Ferris's laboratory (The University of Pittsburg, Pittsburg, PA) in 2011. FaDu and Cal27 were purchased from ATCC in 2014. 1483 was obtained in 2007 from Gary Clayman's laboratory (MD Anderson Cancer Center). The human lung cancer cell lines A549, H1299, H460, H1703, H157, PC-9, and H1975 were obtained from Dr. Sun's laboratory (Emory University). HNSCC cell lines were grown in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum (FBS) and human lung cancer cell lines were grown in RPMI supplemented with 10% FBS. These cells were incubated at 37℃ and 5% CO_2 humidified environment. Media was changed every 3 days and sub-cultured based on confluency. The authenticity of all cell lines was verified through genomic short tandem repeat (STR) profiling by the Research Animal Diagnostic Laboratory, University of Missouri (Columbia, MO) in September 2009, and by the Emory University Integrated Genomics Core (EIGC) in October 2014. A549 and H460 cells-expressing shp53 were previously established as described (Amin et al., 2009).

Protocols for cell culture

Heat Inactivation of FBS

- Frozen FBS was left overnight at 2-8℃ in the refrigerator after which it was completely thawed at room temperature.
- A water bath with sufficient water for serum immersion was prepared and brought to 56℃ for the heat inactivation process.
- Serum was placed in the water bath and a timer was set to 30 minutes once the temperature was steady at 56℃.
- Serum was gently swirled every 3-5 minutes to ensure heat distribution throughout the process.
- After 30 minutes, the flask was gently swirled and the bottle was allowed to cool to room temperature.
- Finally, 50 ml aliquots were stored at -20℃.

Making complete media

- Flasks containing the medium and heat-inactivated FBS were placed in a water bath set to 37℃.
- After equilibration, the flasks were taken out of the water bath and sprayed at the neck with 70% ethanol before transfer to the cell culture hood.
- The appropriate volume of FBS required to make media enriched with 10% FBS was added to the medium and mixed by up-down rotation.

Reviving cells from liquid nitrogen

- The media prepared above were used for reviving frozen cells.

- Cryo-vials containing frozen cell lines were taken out of liquid nitrogen (-185℃) and thawed quickly by placing in a water bath at 37℃ for 1 minute with constant agitation or by rubbing between palms.
- The cell suspension was added dropwise to a 15ml tube containing 5ml complete media.
- The resulting mixture was centrifuged for 3-4 minutes at 1200 rpm, the supernatant liquid was carefully sucked and 5ml media was used to resuspend the pellets.
- The cell suspension was transferred to a 25 cm^3 flask and incubated at 37℃ with 5% CO2, media was changed every 3 days.

Subculturing cells

- When cultured cells had reached 70% confluency, media was sucked from the flask and rinsed with 5ml phosphate-buffered saline (PBS).
- PBS was sucked and the cells were trypsinized with 0.5-1ml 0.25% trypsin-EDTA depending on the size of the flask ensuring that trypsin was evenly spread to all corners of the flask.
- Flasks were incubated at 37℃ for 1-3 minutes.
- The cells were then examined under a microscope to confirm the detachment of adherent cells.
- 5ml fresh serum-containing media was used to resuspend cells in the flask and inactivate the trypsin.
- The cell suspension was transferred into a 15ml tube and centrifuged for 3 minutes at 1200 rpm.

- The supernatant liquid was sucked, and cells were resuspended in a 10ml culture medium. 2.5ml was transferred to a 75 cm^3 flask containing about 10ml of complete media, mixed well, and transferred into the incubator.

Preparing cell stock

- Cultured cells were rinsed with PBS, trypsinized with 0.25% trypsin-EDTA, and incubated for 1-3 minutes for detachment.
- The cells were then observed under the microscope to confirm detachment and resuspended in 5-10ml of media and collected in 15ml tubes.
- The suspended cells were centrifuged for 3-4 minutes at 1000-1200 rpm.
- The supernatant media was sucked, and cells were resuspended in 9ml FBS. 1ml DMSO was added dropwise and mixed by up-down pipetting. This made a 90% FBS + 10% DMSO suspension.
- 1ml of the cell suspension was added to each cryo-vial and transferred to -80℃ for 2-3 days in a cryo-box before final storage in liquid nitrogen (-185℃).

SRB Assay for Growth Inhibition

Growth inhibition and IC_{50} determination were assessed using the sulforhodamine (SRB) assay technique as stated below:

- 100uL cell suspension of about 2,500 cells was cultured in each well of a 96-well plate and incubated overnight for attachment.
- 100uL drug solution (different concentrations to cover a wide range, quadruple for each concentration) was added to each well.
- After incubation for 72 hours, the cells were fixed with cold 10% Trichloroacetic acid (TCA) for 1 hour at 4℃.

- Fixed cells were washed 5 times with double distilled water and air-dried overnight.
- Cells were then stained with 50uL of SRB for 10 minutes at room temperature and washed 5 times with 1% acetic acid and air-dried overnight.
- Bound dye was dissolved with 100uL 10mM Tris solution (pH 10) and absorbance/ optical density was read at 492nm.
- Survival percentage was calculated from the optical density readings using the formula:

  Survival % = (Mean OD treatment/Mean OD control) ×100%

  % growth inhibition = 100 – Survival %

- CalcuSyn software was used to determine the growth curve and IC_{50}.

A flowchart of the SRB assay is provided in Appendix C.

Apoptosis Assay

The cytotoxic effect of actinomycin D was measured by analyzing apoptotic cells using Annexin V-PE/ 7AAD (BD Biosciences) apoptosis assay using the following protocol:

- About 150,000 cells/well of a 6-well plate in 3ml of media were incubated in a CO_2 incubator overnight.
- The following day, the media was replaced with fresh media, and the cells were treated with different concentrations of actinomycin D for different durations/ timepoints.
- After incubation for the desired length of time, the cells were trypsinized and washed with 1ml of 1x PBS.
- The washed cells were centrifuged and resuspended in 1x binding buffer (100uL).
- 5uL Annexin V-PE and 5uL of 7-AAD were added to the cells and incubated at room temperature for 15 minutes in the dark.

- Finally, stained cells were analyzed using ACEA® flow cytometer. The excitation and emission wavelengths were 488nm and 525nm respectively. The number of cells counted was 30,000.
- The data were analyzed by FlowJo software to quantify apoptosis.

Western Blot

The western blot experiments included in this thesis were conducted using the following protocols.

Protein Extraction

- 10ul/ml of protease inhibitor cocktail (Thermo Scientific) was added to lysis buffer (1M Tris-HCl, 5M NaCl, 20% Sodium Azide, Na-Deoxycholate, Igepaland 20% SDS) to make a working solution.
- Cells were lysed over ice using 300ul of working lysis buffer and a cell scrapper.
- The lysates were vortexed at intervals of 10 minutes for 15sec over 1 hour.
- Then the lysates were centrifuged at 12000rpm for 15minutes. The supernatant was collected and stored at -80°C.

Protein Assay

Protein quantification assays were conducted using the Pierce™ BCA Protein Assay Kit. The protocol for this assay is outlined below:

- To generate a standard curve, Bovine Serum Albumin (BSA) solutions covering a range of concentrations (2mg/ml - 0.125mg/ml) were prepared by dilution with lysis buffer. This was used to generate a standard curve.
- The working reagent was prepared by mixing reagents A and B in a ratio of 50:1.
- 190uL working reagent was added to each well of the microplate.

- Two replicates (10uL each) of the standard and sample proteins were added to their respective wells containing the working reagent.
- After mixing by light shaking, the microplate was incubated for 30mins at 37℃.
- Absorbance was measured at 562nm in the SPECTRA max 340PC microplate reader
- The protein concentration of the samples was extrapolated from the standard curve.

SDS-PAGE & Western Blotting

- 12% sodium dodecyl sulfate (SDS) polyacrylamide gel was prepared according to the recipe using H_20, 30% Bis-acrylamide solution, 4x separating gel solution, 10% APS and Temed.
- Mini-PROTEAN Tetra Cell kit was used for electrophoresis.
- The inner gasket chamber was filled to the brim with 1x running buffer (containing Tris, Glycine, and SDS) while the outer chamber was filled with 1x running buffer till it reached the indicated mark for two gels.
- Protein samples were mixed with 2x/4x Laemmli sample buffer enriched with β-mercaptoethanol and denatured on a heat-block at 95℃ for 5mins.
- The equal amount of protein from the samples was then loaded onto the gel for electrophoresis.
- Polyacrylamide gel electrophoresis was conducted at 60mA till the sample buffer eluted from the gel.
- When the run was complete, the gels were stacked between filter papers and PVDF membrane (Bio-Rad Immun-Blot® PVDF Membranes for Protein Blotting).
- The proteins were blotted onto the gel using the blotting electrodes in transfer buffer with the constant voltage set at 70V for 2 hours.

- After blotting, the PVDF membrane was blocked with 5% nonfat skimmed milk for 1 hour at room temperature.
- The membranes were then hybridized overnight with appropriately diluted primary antibody at 4°C.
- The membrane was washed at least 5 times over one hour with TBST wash buffer.
- The membrane was then hybridized with appropriate secondary antibodies diluted in 5% milk for one hour.
- Finally, the membranes were washed for another hour at least 5 times with TBST wash buffer.
- The washed membranes were treated with chemiluminescence substrate solution (GE Healthcare UK Limited) for 3-5 minutes and visualized using a FluoroChem E imager.

Quantitative Polymerase Chain Reaction (qPCR)

The qPCR experiments were conducted using the following protocols below.

RNA Extraction

- Total RNA was extracted using the Qiagen RNeasy® Mini kit following the manufacturer's instructions.
- The concentration of RNA in each sample was measured and diluted with RNase-free H_2O to the required concentration for qPCR experiments and stored at -80°C.

Real-Time qPCR

- The iTaq Universal SYBR Green One-Step Kit (Bio-Rad) was used for qPCR experiments.
- The reaction mix preparation and thermal cycling protocol were strictly followed according to the manufacturer's instructions.

- The primers used for these experiments were procured from Integrated DNA Technologies. The primer sequences are shown below in table 2. The final primer concentration used was 50nM in a 20uL reaction.
- For each RNA sample, GAPDH was used as the housekeeping gene to normalize target gene mRNA quantification.
- Using an Applied Biosystems Quant Studio 3 thermal cycler, we conducted RT qPCR in a one-step experiment that included reverse transcription (10mins at 50℃), polymerase activation, and DNA denaturation (1min at 95℃), and amplification for 40 cycles.

Table 2: Primer sequences of the genes analyzed in this study.

Gene	Forward	Reverse
GAPDH	5'-TGCACCACCAACTGCTTA-3'	5'-GGATGCAGGGATGATGTTC-3'
p21	5'-TCAGAGGAGGTGAGAGAGCG-3'	5'-CGCAGAAACACC TGTGAACG-3'
PUMA	5'-TGACCACTGGCATTCATTTGG-3'	5'-CCTCCCTCTTCCGAGATTTCC-3'
p27	5-AACGTGCGAGTGTCTAACGG-3'	5-TTGCCCTCTAGGGGTT TGTG-3'

Statistical Analysis

All data were obtained in three independent experiments and expressed as Mean ± SD. A two-way ANOVA test coupled with Tukey's post hoc multiple comparisons test was used to analyze the data and a p-value of < 0.05 was accepted as statistically significant.

CHAPTER 4

RESULTS

Actinomycin D Inhibited Growth of Aerodigestive Tract Cancer Cell Lines

While ACTD is an established antitumor agent especially in pediatric tumors like Ewing's sarcoma and Wilms tumor (Gaspar et al., 2015; van den Heuvel-Eibrink et al., 2017), its activity in aerodigestive tract cancers requires further investigation. We conducted experiments to explore the sensitivity of aerodigestive tract cancer cells to increasing doses of actinomycin D using the SRB assay. Tables 3&4 show the IC_{50} of ACTD against various NSCLC and HNSCC cell lines, respectively measured after 72h treatments. Fadu and H1703 cells were the most and least sensitive cell lines with IC_{50} values of 0.021 and 2.96nM, respectively.

Table 3: IC_{50} values of Actinomycin D against NSCLC cell lines.

Lung Cancer Cell Lines	**IC_{50} (nM)**	**95% CI**	
		Upper	*Lower*
H1975	0.16	0.25	0.1
PC9	0.41	0.55	0.30
H460	0.42	0.63	0.29
A549	1.3	1.72	0.97
H1299	0.65	0.80	0.53
H1703	2.96	5.1	1.70
H157	0.67	0.84	0.53

Table 4: IC_{50} values of Actinomycin D against SCCHN cell lines

HNSCC Cell Lines	IC_{50} (nM)	95% CI	
		Upper	*Lower*
MDA686TU	1.29	1.77	0.91
Fadu	0.02	0.08	0.004
1483	0.65	0.97	0.52
Cal27	0.54	1.39	0.21
SCC47	0.29	0.49	0.18
VU-147T	1.24	0.85	1.80

Actinomycin D Caused Dose- and Time-Dependent Apoptosis in Aerodigestive Tract Cancer Cell Lines

After confirming the growth inhibition by actinomycin D, we assessed the ability of the anti-tumor antibiotic to induce apoptosis on different aerodigestive tract cancer cell lines. Cell lines were selected based on diverse p53 status. Irrespective of p53 status, actinomycin D induced a dose- and time-dependent apoptosis of all cell lines tested (Fig. 6-8).

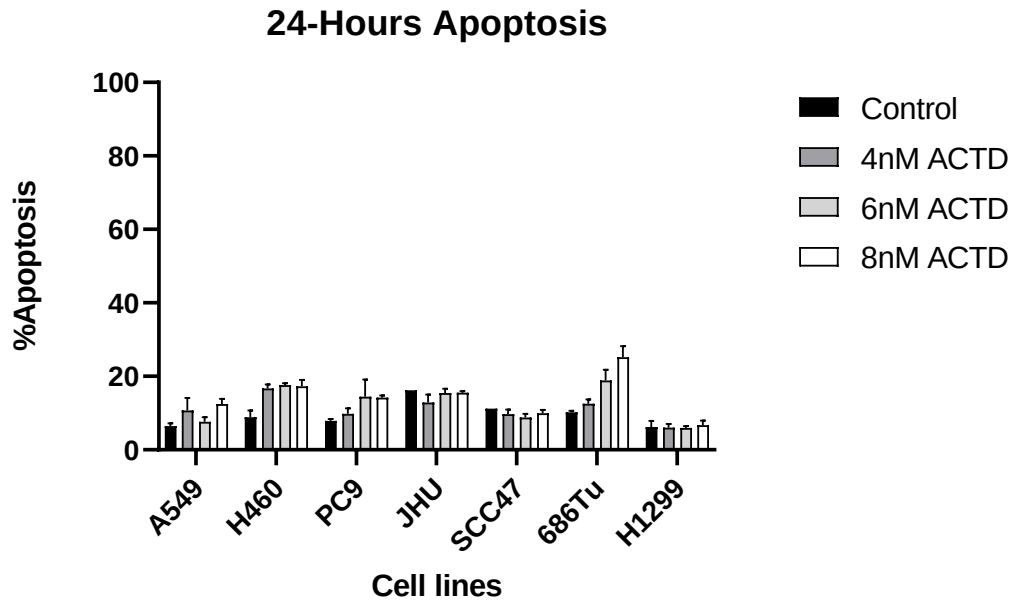


Figure 6: Actinomycin D induced apoptosis at 24 h.

Cells were treated with various doses of actinomycin D for 24 h and apoptosis was measured by annexin V-PE staining. Error bars represent standard deviation from the mean of triplicate treatments. Representative scatter plots are available in appendix B.

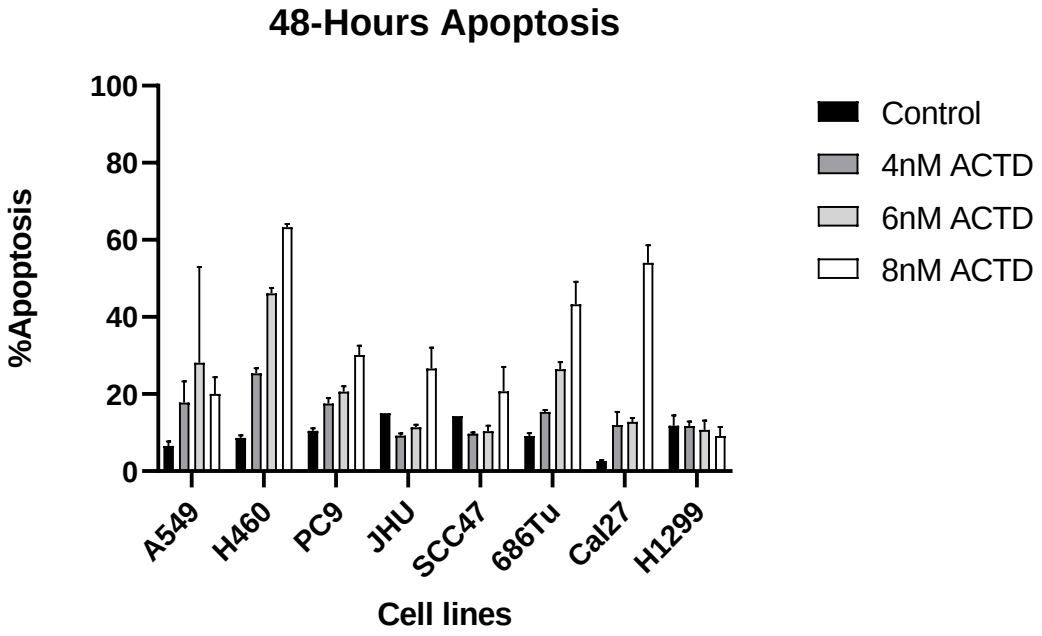


Figure 7: Actinomycin D induced apoptosis at 48 h.

Cells were treated with various doses of actinomycin D for 48 h and apoptosis was measured by annexin V-PE staining. Error bars represent standard deviation from the mean of triplicate treatments. Representative scatter plots are available in appendix B.

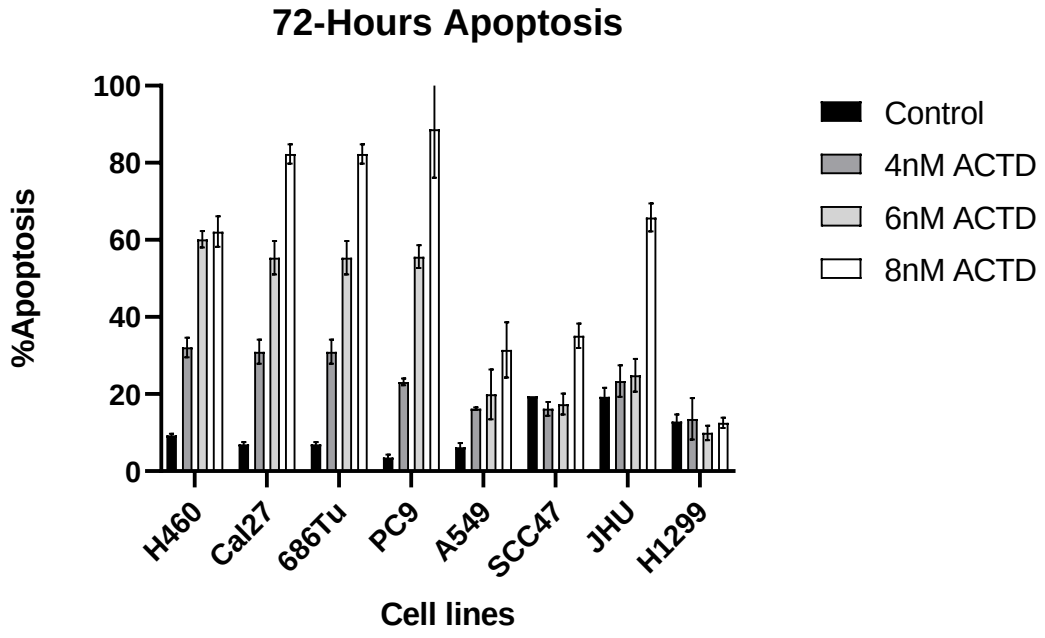


Figure 8: Actinomycin D induced apoptosis at 72 h

Cells were treated with various doses of actinomycin D for 72 h and apoptosis was measured by annexin V-PE staining. Error bars represent standard deviation from the mean of triplicate treatments. Representative scatter plots are available in appendix B.

The quantified apoptosis was confirmed through biochemical experiments which assessed caspase 3 and Poly (ADP-ribose) Polymerase (PARP) cleavage in two cell lines (A549 and H460) after treatment with ACTD. The results show that actinomycin D increased PARP and caspase 3 cleavage in H460 and A549 cells (Fig. 9&10)

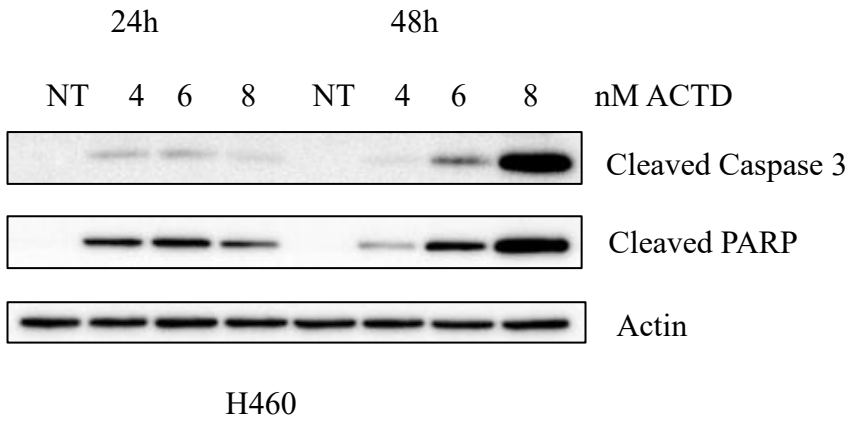


Figure 9: Cleavage of caspase 3 and PARP by actinomycin D in H460 cells.

H460 cells were treated with indicated doses of actinomycin D for 24 and 48 h. Expression of cleaved caspase 3 and PARP was examined in whole-cell lysate by western blotting. Data are representative of three independent experiments.

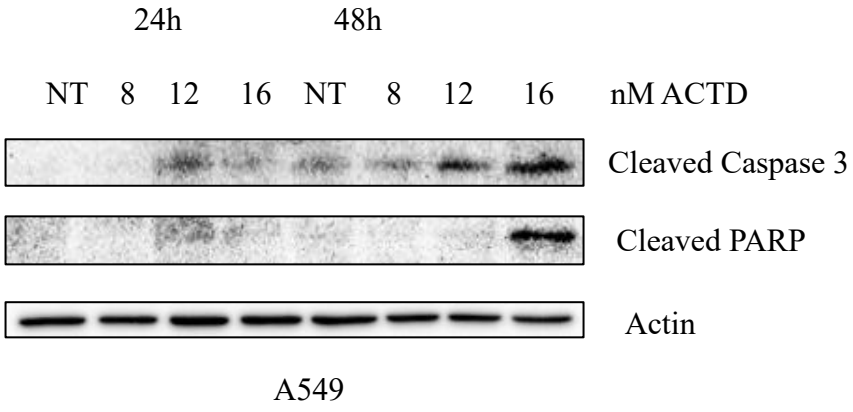


Figure 10: Cleavage of caspase 3 and PARP by actinomycin D in A549 cells.

A549 cells were treated with indicated doses of actinomycin D for 24 and 48 h. Expression of cleaved caspase 3 and PARP was examined in whole-cell lysate by western blotting. Data are representative of three independent experiments.

Dose and Time-Dependent Activation of p53 by Actinomycin D in A549 and H460 Cells

p53 is the most important tumor suppressor due to its role in cell cycle arrest, DNA repair, and apoptosis. It is often activated through post-translational modifications by cellular stress signals like DNA damage, oxidative stress, and oncogene activation (Joerger & Fersht, 2016). As a cytotoxic agent, actinomycin D activates cell stress signals that induce p53 expression and apoptotic effects in human colon cancer cells (Choong et al., 2009). Also, depending on the dose of actinomycin D and cell type, it could induce apoptosis through ribosomal or genotoxic stress signals (Kleeff et al., 2000; Perry & Kelley, 1970). To explore the mechanism of actinomycin D-induced apoptosis, we treated A549 and H460 cells, two NSCLC cell lines with wild-type p53, with increasing doses of actinomycin D for 24 and 48 hours. Total cell lysates were used for SDS-PAGE and immunoblotting for the expression of p53, phospho-p53 (Ser15), and its downstream targets, p21, PUMA, and MDM2. In both cell lines, actinomycin D dose- and time-dependently increased expression of p53 and p-p53 (ser15) (Fig. 11 & 12). In a similar pattern, we observed an increased expression of p53 transcriptional target genes (p21, PUMA, and MDM2).

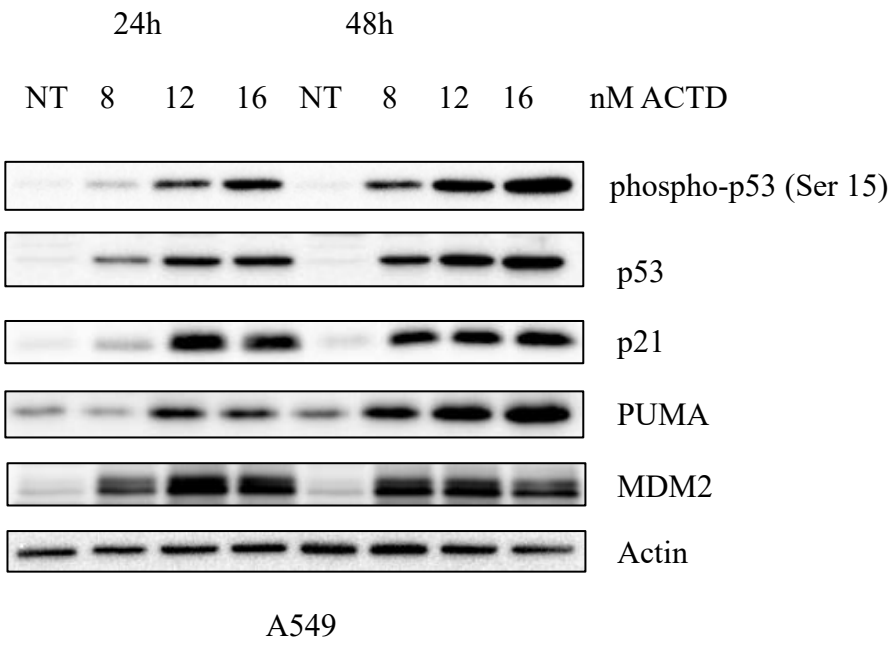


Figure 11: Activation of p53 pathway by actinomycin D in A549 cells.

A549 cells were treated with indicated doses of actinomycin D for 24 and 48 h. Expression of phosphor-p53 (Ser 15), p53, p21, PUMA, and MDM2 were examined in whole-cell lysate by western blotting. Data are representative of three independent experiments.

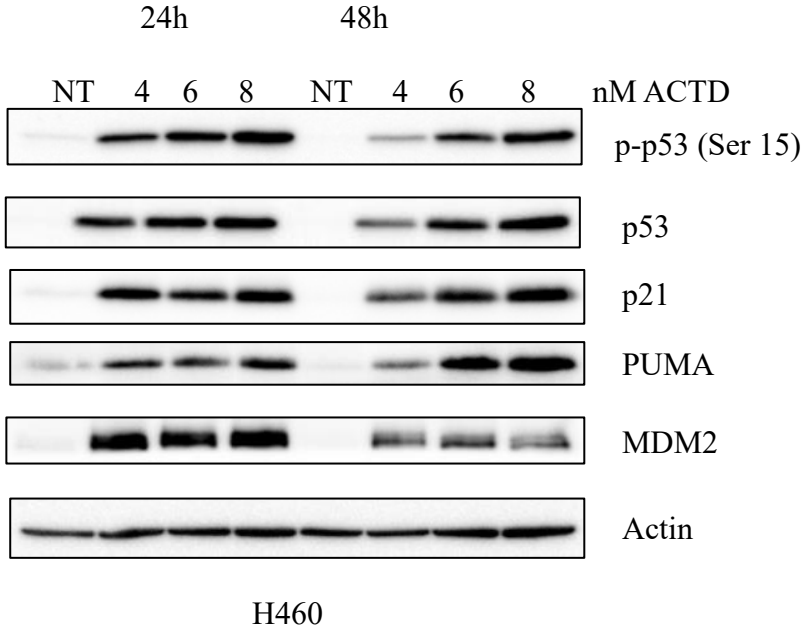


Figure 12: Activation of p53 pathway by actinomycin D in H460 cells.

H460 cells were treated with indicated doses of actinomycin D for 24 and 48 h. Expression of phosphor-p53 (Ser 15), p53, p21, PUMA, and MDM2 was examined in whole-cell lysate by western blotting. Data are representative of three independent experiments.

Actinomycin D Activates p53 Transcriptional Activity in A549 and H460 Cells

As a transcription factor, phosphorylation of p53 at ser 15 would promote the transcription of cell-cycle arrest and DNA repair genes. In the event of significant DNA damage, Ser 46 is also phosphorylated, and the transcription of apoptotic mediators is promoted (Oda et al., 2000; Taira et al., 2007). The results show that actinomycin D phosphorylates p53 at Ser 15 residue in H460 and A549 cells. To confirm that the increased expression of p21 and PUMA was due to transactivation, total RNA was extracted after 24-hour treatment of A549 and H460 cells with actinomycin D. Expression of p21, p27, and PUMA mRNA were examined by qPCR. As shown in Fig. 13 and 14, actinomycin D significantly increased the mRNA expression of p21 and PUMA

in A549 cells whereas only selectively increased the expression of p21 in H460 cells. There was no significant effect on the expression of p27 in both cell lines. These results suggesting that actinomycin D selectively transactivates p53 target genes depending on cell lines.

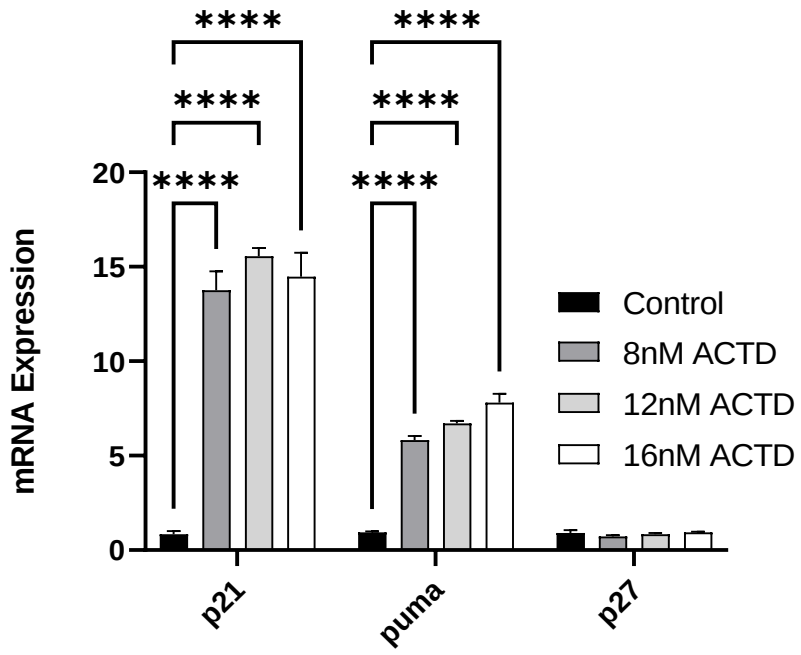


Figure 13: Actinomycin D selectively increases transactivation of p53 target genes in A549 cells.

A549 cells were treated with indicated doses of actinomycin D for 24 h. Total RNA was used for the expression of p21, p27, and PUMA mRNA by qPCR. Error bars indicate SD from triplicate treatments. $P < 0.05$.

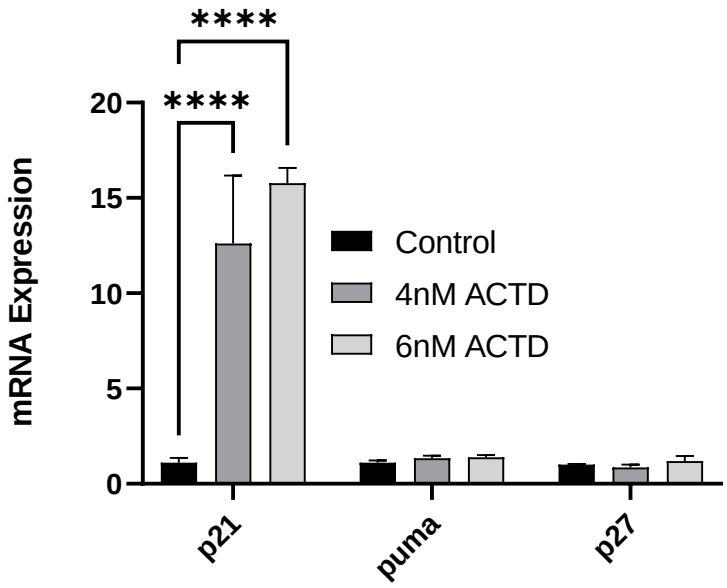


Figure 14: Actinomycin D selectively increases transactivation of p53 target genes in H460 cells.

H460 cells were treated with indicated doses of actinomycin D for 24 h. Total RNA was used for the expression of p21, p27, and PUMA mRNA by qPCR. Error bars indicate SD from triplicate treatments. $P < 0.05$.

Since we observed correlative activation of p53 and expression of p21 and PUMA, we next explored the role of p53 in actinomycin D-induced apoptosis. Previously developed A549 and H460 cells with ablated p53 expression with a lentivirus-based shRNA construct were used (Amin et al., 2010). Parental (wild-type p53) and shRNA knocked-down p53 cells were treated with increasing doses of ACTD for 48 hours. The cell lysates were used to assess the expression of p53 and p53 targets p21 and PUMA. The results show that ablating p53 expression completely abolished the expression of p21 and PUMA in A549 cells (Fig. 15). Interestingly, H460 cells showed a similar pattern with p21 but not PUMA, which was significantly expressed in the H460 shp53 cells (Fig. 16). Indeed, actinomycin D did not increase the expression of PUMA mRNA in

H460 cells (Fig 14) suggesting that the increased PUMA expression by actinomycin D in H460 cells is posttranscriptional.

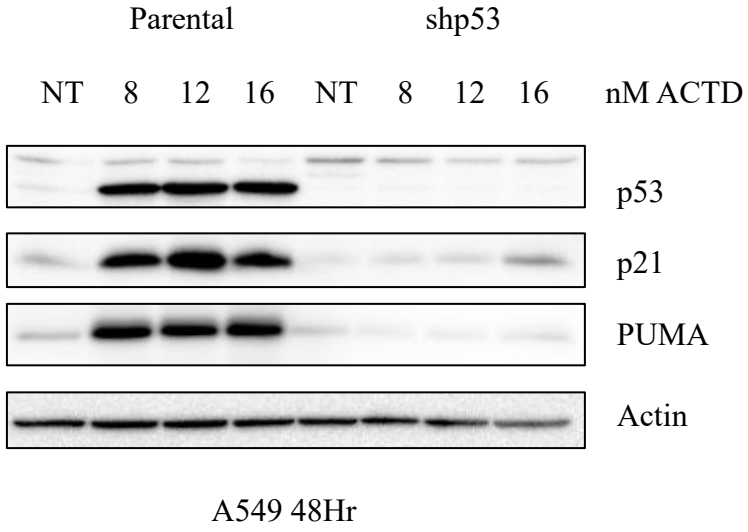


Figure 15: p53-dependent expression of p21 and PUMA by actinomycin D in A549 cells. Parental and p53 ablated cells were treated with indicated doses of actinomycin D for 48 h. Expression of p53, p21, and PUMA were examined in total cell lysates by western blotting. Data are representative of three independent experiments**.**

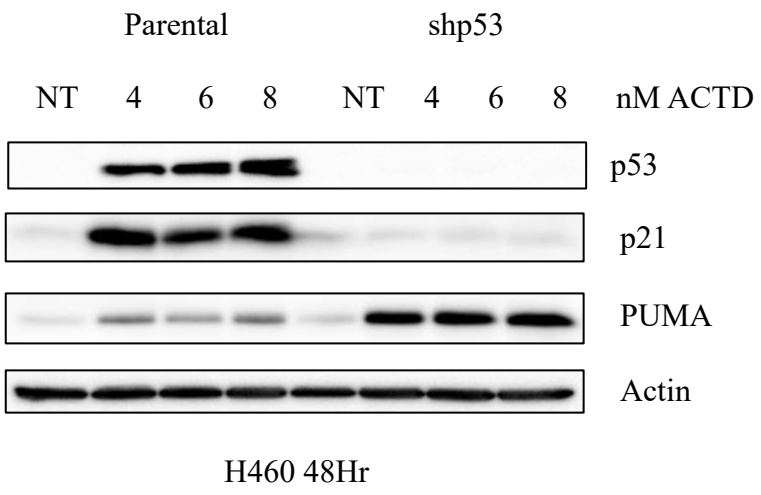


Figure 16: p53-dependent expression of p21 but not PUMA by actinomycin D in H460 cells.

Parental and p53 ablated cells were treated with indicated doses of actinomycin D for 48 h. Expression of p53, p21, and PUMA was examined in total cell lysates by western blotting. Data are representative of three independent experiments.

Interestingly, in our qPCR studies, we observed a significant difference in the expression of actinomycin D-induced p21 mRNA in both H460 and A549 parental cells when compared to cells with shp53 (Fig. 17 and 19). In the case of Puma, we found significant inhibition of Puma mRNA expression after ablation of p53 in the A549 pair (Fig. 18). However, in H460, similar to protein expression, Puma mRNA expression was not affected by ablation of p53 (Fig. 20). These results suggest that although actinomycin D-induced expression of p21 is p53-dependent in both A549 and H460 cells, the expression of Puma is cell line-specific; dependent on p53 in A549 cells while independent of p53 in H460 cells.

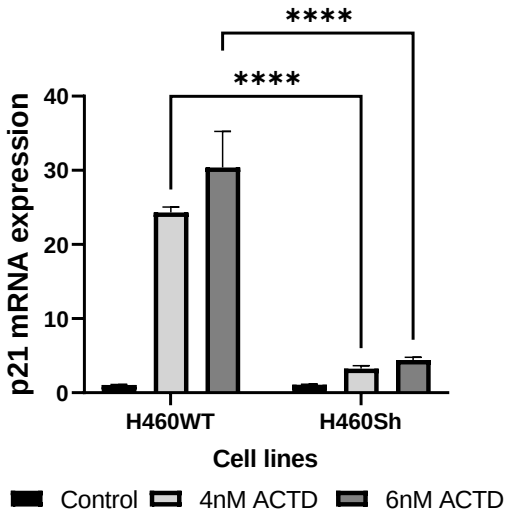


Figure 17: Actinomycin D-induced p21 transactivation is p53-dependent in H460 cells.

Parental and p53 ablated H460 cells were treated with different doses of actinomycin D for 24 h. Expression of p21 mRNA was examined in total RNA by qPCR.

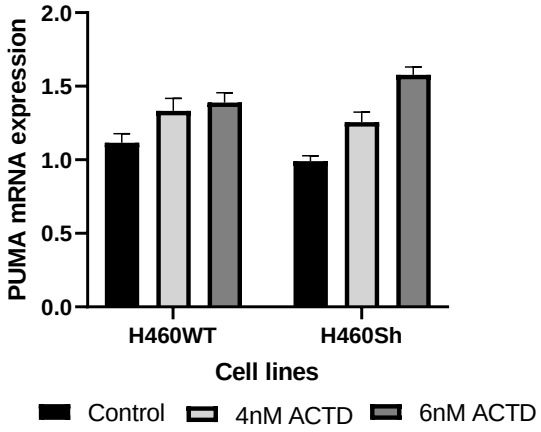


Figure 18: Actinomycin D has no effect on PUMA mRNA expression in H460 cells.

Parental and p53 ablated H460 cells were treated with different doses of actinomycin D for 24 h. Expression of PUMA mRNA was examined in total RNA by qPCR.

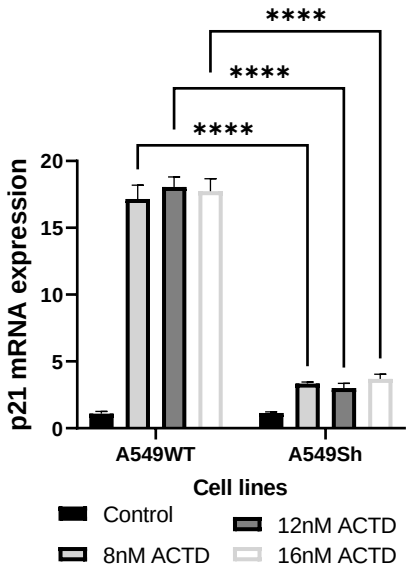


Figure 19: Actinomycin D-induced p21 transactivation is p53-dependent in A549 cells.

Parental and p53 ablated A549 cells were treated with different doses of actinomycin D for 24 h. Expression of p21 mRNA was examined in total RNA by qPCR.

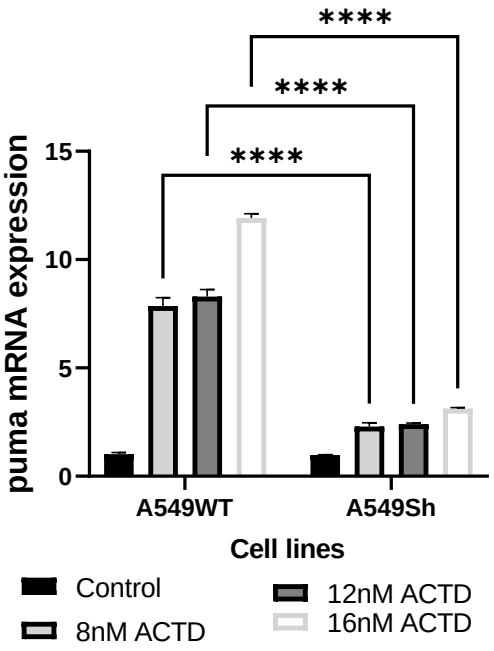


Figure 20: Actinomycin D-induced PUMA transactivation is p53-dependent in A549 cells.

Parental and p53 ablated A549 cells were treated with different doses of actinomycin D for 24 h. Expression of PUMA mRNA was examined in total RNA by qPCR.

Furthermore, we observed a significant difference in Annexin-V quantified apoptosis between cells with wild type p53 and their shp53 knockdown counterparts after 48 hours of treatment with Actinomycin D (Fig. 21 & 22).

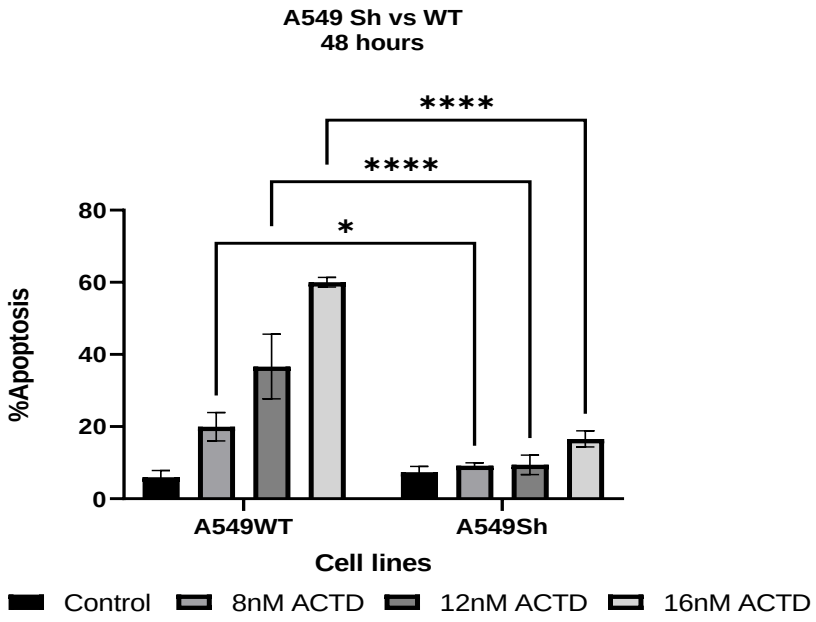


Figure 21: Actinomycin D-induced p53-dependent apoptosis in A549 cells.

Parental and p53 ablated cells were treated with different doses of actinomycin D for 48 h. Apoptosis was measured by annexin V-PE staining. Error bars indicate SD from triplicate treatments.

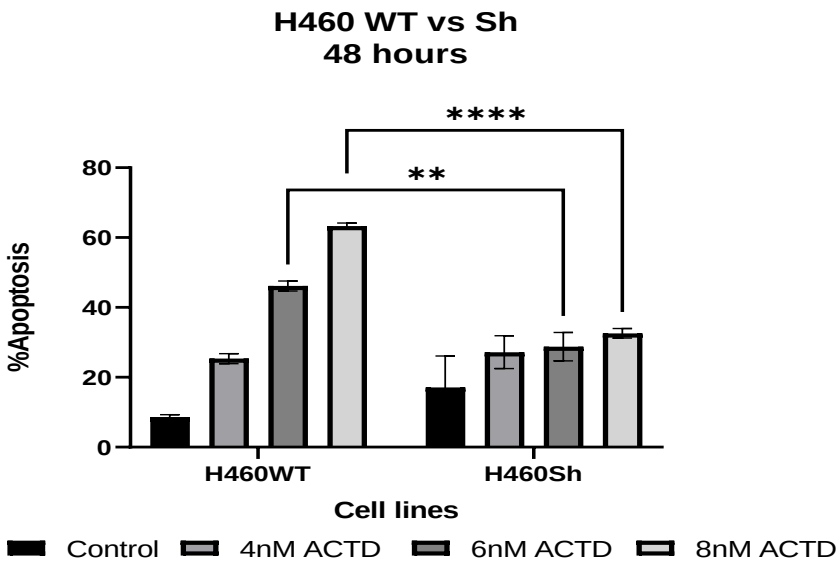


Figure 22: Actinomycin D-induced p53-dependent apoptosis in H460 cells.

Parental and p53 ablated cells were treated with different doses of actinomycin D for 48 h. Apoptosis was measured by annexin V-PE staining. Error bars indicate SD from triplicate treatments.

DNA Damage is not a Major Trigger for Actinomycin D-Induced p53 Activation

Next, we evaluated the mechanism by which actinomycin D induced p53 activation. Previous research has shown that actinomycin D binds to DNA and inhibits DNA-dependent RNA synthesis (Koba & Konopa, 2005). Also, several forms of stress signals within a cell could activate p53 and DNA damage is one of such stressors. Therefore, we evaluated DNA damage as a possible mechanism by which actinomycin D activated p53. A549 and H460 cells were treated with increasing doses of actinomycin D for 24 and 48 hours and examined the expression of pH2AX, a marker of DNA double-strand break. The phosphorylation of H2AX is essential for DNA damage response and the recruitment of DNA repair proteins, which makes it a good monitor for genotoxic effects (Podhorecka et al., 2010). The results showed that actinomycin D had very little effect on

H2AX phosphorylation (Fig. 23 and 24), Only a high dose and longer time of treatments had some effect suggesting that actinomycin D activates p53 without significant double-strand breaks.

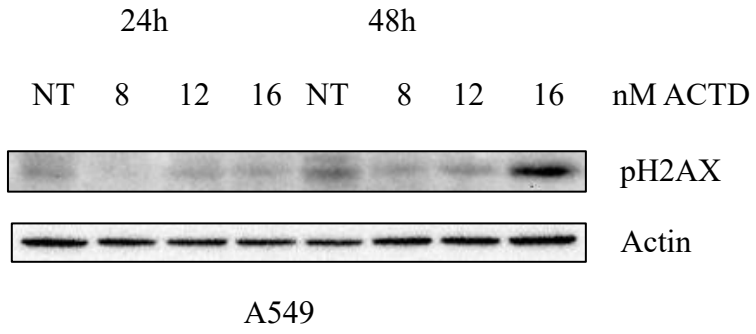


Figure 23: Actinomycin D-induced DNA damage in A549 cells.

A549 cells were treated with different doses of actinomycin D for 24 and 48 h and phosphorylation of H2AX was examined in whole-cell lysates by western blotting.

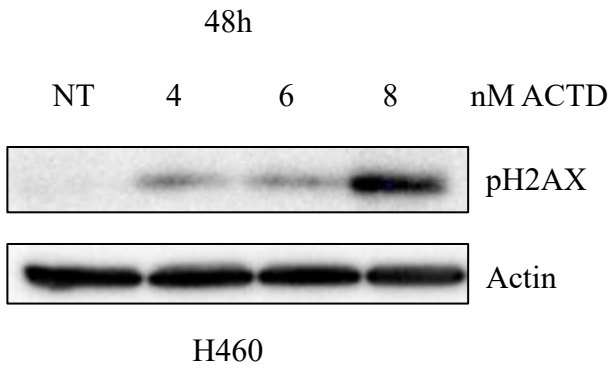


Figure 24: Actinomycin D-induced DNA damage in H460 cells.

H460 cells were treated with different doses of actinomycin D for 48 h and phosphorylation of H2AX was examined in whole-cell lysates by western blotting.

Mechanism of Actinomycin D-Induced Apoptosis in Cells Lacking Functional p53

Our results confirmed dose- and time-dependent increase in apoptosis irrespective of p53 status (Fig 6-8). So, after confirming the role of p53 in actinomycin D mediated apoptosis, it was imperative to explore the mechanism of apoptosis in cells with mutated p53. There is mounting

evidence that in the absence of functional p53, other p53 family members like p73 can mediate apoptosis (El Dika, 2020; Ramos et al., 2020). To evaluate this, we treated PC9 cells with increasing doses of actinomycin D for 24 and 48 hours. Cells were lysed and immunoblotted for Caspase 3 and PARP to confirm apoptosis (Fig 25). We also assessed the expression of p73 and traditional p53 downstream targets, p21, PUMA, and MDM2. The results show an increase in p73 protein expression after actinomycin D treatments. Similarly, downstream transcriptional targets like p21, PUMA, and MDM2 were upregulated (Fig 26).

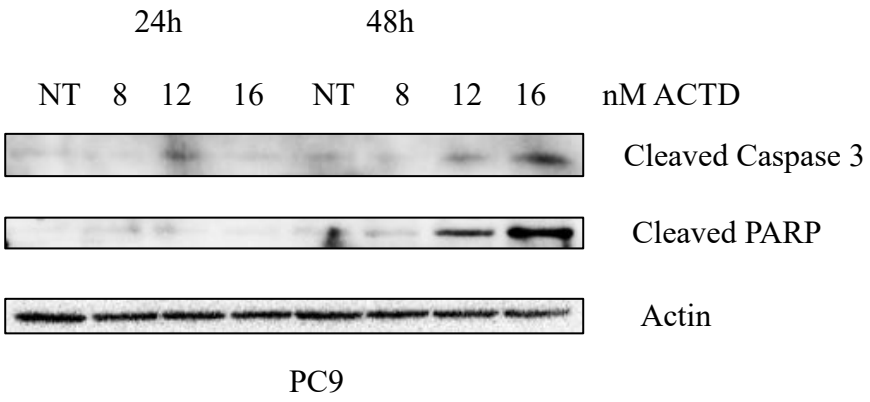


Figure 25: Cleavage of Caspase3 and PARP by actinomycin D in PC9 cells.

PC9 cells were treated with different doses of actinomycin D for 24 and 48 h and expression of caspase 3 and cleaved PARP were examined in whole-cell lysates by western blotting.

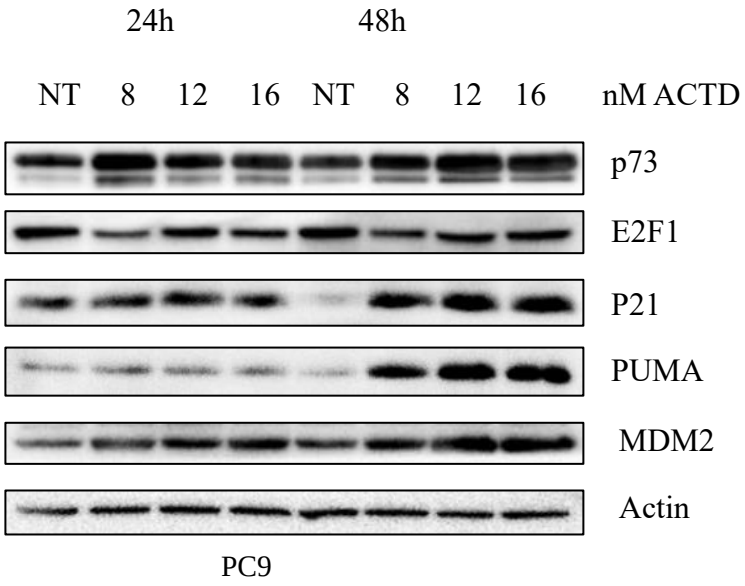


Figure 26: Activation of p73/E2F1 pathway by actinomycin D in PC9 cells.

PC-9 cells were treated with different doses of actinomycin D for 24 and 48 h and expression of p73, E2F1, p21, PUMA, and MDM2 were examined in whole-cell lysates by western blotting.

Differential Expression of p53-Target Genes by Actinomycin D in Different Cell Lines

In our mRNA studies with the wild type p53 cells (A549 and H460), we observed that Actinomycin D selectively transactivated p53 targets genes depending on the cell line (Fig. 13 & 14). However, p21, which is a p53 mediator for cell cycle arrest, was significantly upregulated in both cell lines. It was important to determine if this effect was consistent in p53 wild type and mutant settings. So, we evaluated the effect of low dose Actinomycin D on the p53 target genes expression in aerodigestive tract cancer cells with wild type and mutant p53. Total RNA was extracted after 24-hour treatment of A549, H460, and PC9 cells with 4nM actinomycin D. Expression of p21, p27, and PUMA mRNA were examined by qPCR. As shown in Fig. 27 low dose actinomycin D selectively increased the mRNA expression of p21 in A549 and H460 cells with wild type p53 but not in PC9 cells with mutant p53.

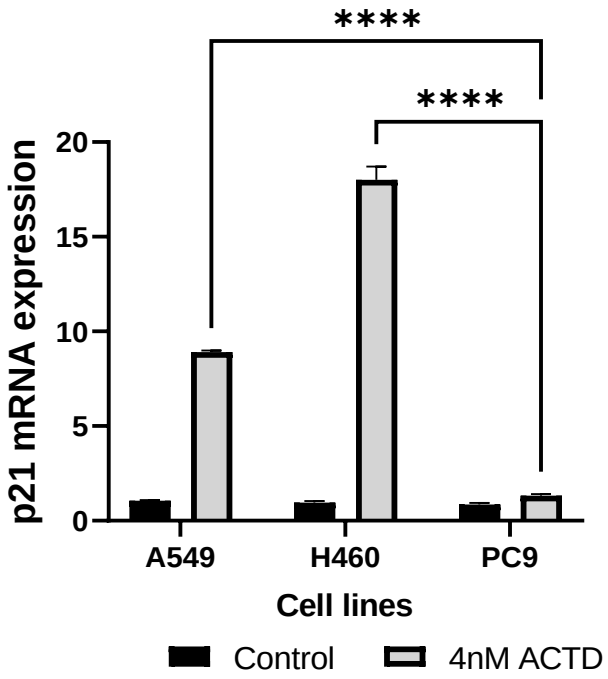


Figure 27: Low dose actinomycin D significantly upregulates p21 in wild type p53 cells. A549, H460, and PC9 cells were treated with 4nM of actinomycin D for 24 h. Total RNA was used for the expression of p21 mRNA by qPCR. Error bars indicate SD from triplicate treatments. $P < 0.05$.

Similarly, low-dose actinomycin D selectively upregulated PUMA in A549 and H460 cells but not PC9 cells (Fig. 28).

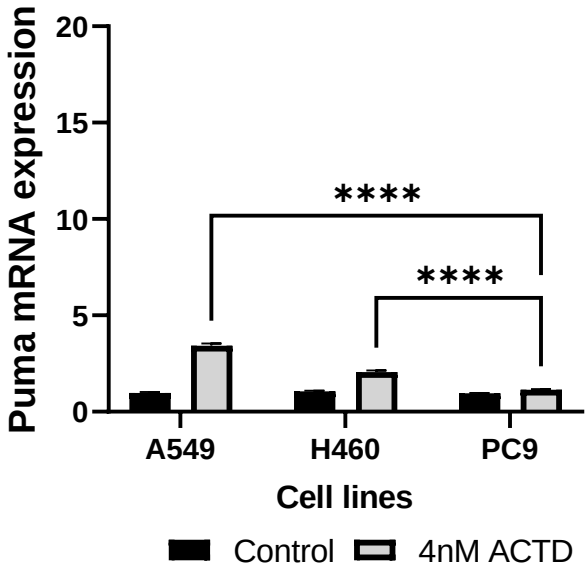


Figure 28: Low dose actinomycin D significantly upregulates PUMA in wild type p53 cells. A549, H460, and PC9 cells were treated with 4nM of actinomycin D for 24 h. Total RNA was used for the expression of PUMA mRNA by qPCR. Error bars indicate SD from triplicate treatments. $P < 0.05$.

Interestingly, low dose Actinomycin D had a negligible effect on p27 mRNA expression in the three cell lines irrespective of p53 status (Fig. 29). These findings suggest that low dose actinomycin D induces a context-dependent differential expression of p53 target genes, especially a significant upregulation of p21 in p53 competent cells, which could be beneficial to the application of low dose actinomycin D as a chemoprotectant in p53-mediated cyclotherapy for aerodigestive tract cancers.

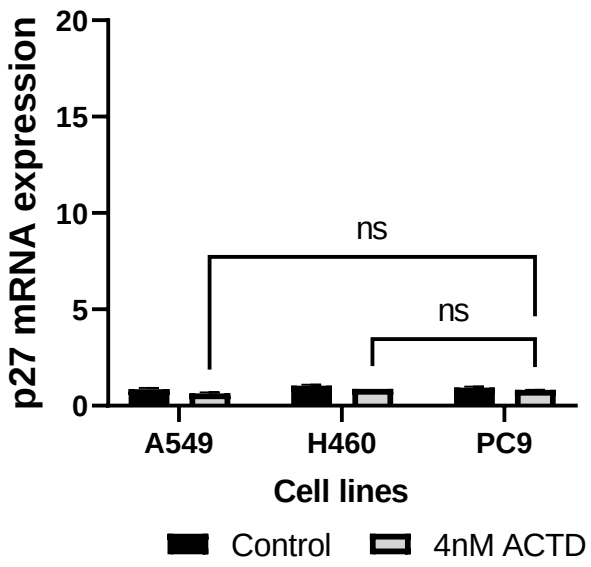


Figure 29: Low dose actinomycin D has a negligible effect on p27 mRNA expression in aerodigestive tract cancer cells irrespective of p53 status.

A549, H460, and PC9 cells were treated with 4nM of actinomycin D for 24 h. Total RNA was used for the expression of p27 mRNA by qPCR. Error bars indicate SD from triplicate treatments. $P < 0.05$.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

The management of aerodigestive tract cancers, which include HNC and lung cancers is rife with challenges, especially resistance to therapy. Despite several advances in oncology and the discovery of new targeted therapies and immunotherapies, the prognosis for these cancers is still poor, especially with lung cancer (Woodard et al., 2016). However, recent research has shown that low-dose actinomycin D could be effective against resistant cancers when combined with other therapy options by targeting cancer stem cells (Green et al., 2019). More so, it could reduce the toxicity of cytotoxic chemotherapy to normal cells through cyclotherapy (Rao et al., 2013). To apply these strategies to aerodigestive tract cancers, as a first step, this study aimed to evaluate the apoptotic mechanisms of low-dose actinomycin D in aerodigestive tract cancers.

The sensitivity of cancer cells to actinomycin D-induced growth inhibition may be context-dependent. In our studies with aerodigestive tract cancer cell lines, actinomycin D inhibited the growth of all cell lines tested, and we obtained IC_{50} values that ranged from 0.02nM -2.96nM, which were comparable to those obtained in ependymoma and hepatocellular cancer cells treated with low dose actinomycin D (Singhal & Rajeswari, 2009; Tzaridis et al., 2016). However, others have also reported slightly higher actinomycin D IC_{50} values up to 2.5uM in glioblastomas (Taylor et al., 2020).

To determine if actinomycin D induced apoptosis in the treated cells, we conducted apoptosis assays using the Annexin V-PE staining. We observed a dose-and time-dependent increase in apoptosis of all cell lines treated with actinomycin D (Fig 6-8), however, sensitivity varied between cells. Interestingly, the H1299 cells which are deficient in p53 had significantly lower apoptosis compared to other cell lines tested. This piqued our interest in exploring the role

of p53 in actinomycin D-induced apoptosis in aerodigestive tract cancers. Previous studies have shown that actinomycin D, as a DNA damaging agent, can activate p53 through a genotoxic stress response, which promotes apoptosis (Choong et al., 2009).

Mechanistic Studies

To determine actinomycin D's molecular mechanism of apoptosis, A549, and H460 (WT p53), and PC9 (mutant p53) cells were used. The quantified apoptosis was confirmed biochemically, by the observed expression of cleaved caspase 3 and PARP (Fig 9 & 10), which are putative markers of apoptosis (Decker & Muller, 2002). As expected, there was a dose-and time-dependent increase in p53 expression after treating wild type A549 and H460 cells. Similarly, the expression of downstream transcriptional targets of p53 involved in growth arrest (p21) and apoptosis (PUMA) increased. However, to confirm that the observed increase was due to transactivation, we assessed the mRNA levels of p21 and PUMA after actinomycin D treatment. Truly, there was a significant increase in the mRNA expression of p53 transcriptional target p21. Expression of PUMA was differential (Fig 13 & 14) after actinomycin D treatment, which indicated actinomycin D treatment activated p53's transcriptional activity in a cell line-specific manner.

p53 activity is regulated through a negative feedback mechanism by one of its transcriptional targets, Mdm2. Knowing this, we assessed the expression of Mdm2 after treatment of A549 and H460 cells with actinomycin D. Unsurprisingly, Mdm2 expression increased in a dose-dependent manner within the first 24 hours but did not affect p53 transcriptional activity in A549 and H460 cells (Fig 11 & 12). Previous evidence had demonstrated Mdm2 upregulation does not inhibit p53 transcriptional activity and cytotoxicity in the presence of DNA damaging agents (McKenzie et al., 2002).

Furthermore, to confirm that actinomycin D caused DNA damage in A549 and H460 cells, we assessed the expression of pH2AX, a known indicator of DNA damage. We observed an increased expression only at the highest doses after 48 hours (Fig 23 & 24). This contradicts previous evidence that actinomycin D intercalates DNA strands leading to double-strand breaks, which leads to rapid induction of p53 (Fontoura et al., 1997; Kastan et al., 1991). A possible and alternative mechanism is the inhibition of RNA polymerase II. At low doses actinomycin D preferentially inhibits ribosomal RNA synthesis, this leads to ribosomal stress which is a known cellular stressor for p53 activation (Choong et al., 2009).

Interestingly, the ablation of p53 significantly decreased the observed apoptosis in A549 and H460 cells (Fig 21 & 22). More so, except for Puma in H460 cells, the protein expression of p53 downstream transcriptional targets was abolished in the absence of p53 (Fig 15 &16). The effect of actinomycin D on the expression of PUMA protein and mRNA was dependent on cell lines. Although the expression of PUMA protein was increased in both A549 and H460 cells, ablation of p53 only inhibits the expression of PUMA in A549 cells. Interestingly, we found that although actinomycin D-induced PUMA mRNA expression in A549 cells, it did not affect PUMA mRNA expression in H460 cells suggesting that the increase in PUMA protein expression in H460 cells is posttranscriptional.

In the absence of p53, E2F1 can induce apoptosis by activating the transcription of PUMA through p73 (Irwin et al., 2000; Stiewe & Pützer, 2000). This prompted our investigation of the apoptotic mechanisms of actinomycin D in mutant p53 cell line (PC9). PC9 cells have a missense R248Q mutation on the TP53 gene, which inactivates the p53 protein (Molina-Vila et al., 2014). Nevertheless, we observed a dose-and-time-dependent increase in apoptosis of PC9 cells treated with actinomycin D. To explain this in the absence of functional p53, we investigated the activation

of alternative pathways involving p73. As a homolog of p53, p73 can transcriptionally activate traditional p53 targets like p21, Mdm2, GADD45, and induce apoptosis irrespective of contextual p53 functionality (Ray et al., 2011). We observed that actinomycin D treatment in PC9 cells increased the protein expression of p73, p21, and PUMA (Fig 26). However, some studies have also reported that tumor-derived p53 mutants could inactivate the p73/E2F1 pathway by interacting with p73 (Di Como et al., 1999; Stiewe & Pützer, 2000). Truly, our mRNA expression data showed that the increased protein expression of p21 and PUMA may not be due to transactivation by p73 (Fig 27-29).

Based on these findings, we can conclude that actinomycin D exerts apoptosis via p53-dependent and –independent mechanisms. These results will guide future combinatorial studies involving actinomycin D.

Future Direction

As a potential chemoprotectant in p53-mediated cyclotherapy, previous studies have shown that low-dose Actinomycin D could exert sub-lethal doses of DNA damage that cause reversible G1/G2 cell cycle arrest in normal cells with wild type p53 (Blagosklonny, 2002; Rao et al., 2010; van Leeuwen et al., 2012). This serves as a protective mechanism for normal cells upon the addition of S or M phase-specific chemotherapeutics (Rao et al., 2010). In our present study, we observed that low dose actinomycin D induced differential expression of p53 target genes but consistently upregulated the expression of p21 in cells with wild-type p53. Based on this knowledge, we can propose that low-dose actinomycin D would induce reversible cell cycle arrest to protect normal cells with wild-type p53. Cell cycle analysis studies are needed to evaluate this hypothesis. This would inform the selection of complementary chemotherapeutics for the potential application of low-dose actinomycin D in cyclotherapy for aerodigestive tract cancers.

To confirm transcription-independent apoptosis in mutant p53 cell lines, future studies could assess the expression of p53 S392 phosphorylation or the upregulation of Bax protein, which has also been shown to be activated by a transcription-independent fashion in mutant p53 cells (Castrogiovanni et al., 2018; Speidel et al., 2006).

Some studies have reported that the S392A mutant p53 in HCT-116 cells retained some of the normal post-translational modifications and transcriptional activities of wildtype p53 after exposure to cytotoxic therapy (Castrogiovanni et al., 2018). In our case, PC9 has an R248Q missense mutation on the TP53 gene, which we could have assessed for retention of some traditional p53 functions. But our focus was on an alternative pathway involving p73. Future research can further explore any retention of p53 activity in the R248Q mutant in PC9 cells. More so, transcriptionally impaired p53 mutants have been shown to interact with proapoptotic Bcl-2 protein Bak to induce cytochrome c release in vivo, but this did not lead to apoptosis (Pietsch et al., 2008). So future studies should also include in vivo experiments to validate in vitro results.

We could have gone further to identify the specific isoform p73 that was upregulated in PC9 cells because p73 has a complex nature with several isoforms that oppose the tumor-suppressive effects of the TA-p73 isoform (Ramos et al., 2020). Future research could further define the involvement of p73 in ACTD induced apoptosis of p53 mutant cell lines.

REFERENCES

Aboul-Enein, B., Aboul-Enein, F., & Ross, M. (2012). Smallpox inoculation and the Ottoman contribution: A Brief Historiography. *Texas Public Health Journal*, *64*, 12-14.

Amin, A. R., Khuri, F. R., Chen, Z. G., & Shin, D. M. (2009). Synergistic growth inhibition of squamous cell carcinoma of the head and neck by erlotinib and epigallocatechin-3-gallate: The role of p53-dependent inhibition of nuclear factor-kappaB. *Cancer Prev Res (Phila)*, *2*(6), 538-545. https://doi.org/10.1158/1940-6207.Capr-09-0063

Amin, A. R., Wang, D., Zhang, H., Peng, S., Shin, H. J., Brandes, J. C., Tighiouart, M., Khuri, F. R., Chen, Z. G., & Shin, D. M. (2010). Enhanced anti-tumor activity by the combination of the natural compounds (-)-epigallocatechin-3-gallate and luteolin: Potential role of p53. *J Biol Chem*, *285*(45), 34557-34565. https://doi.org/10.1074/jbc.M110.141135

Ang, K. K., Harris, J., Wheeler, R., Weber, R., Rosenthal, D. I., Nguyen-Tân, P. F., Westra, W. H., Chung, C. H., Jordan, R. C., Lu, C., Kim, H., Axelrod, R., Silverman, C. C., Redmond, K. P., & Gillison, M. L. (2010). Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*, *363*(1), 24-35. https://doi.org/10.1056/NEJMoa0912217

Antonia, S. J., López-Martin, J. A., Bendell, J., Ott, P. A., Taylor, M., Eder, J. P., Jäger, D., Pietanza, M. C., Le, D. T., de Braud, F., Morse, M. A., Ascierto, P. A., Horn, L., Amin, A., Pillai, R. N., Evans, J., Chau, I., Bono, P., Atmaca, A.,... Calvo, E. (2016). Nivolumab alone and nivolumab plus ipilimumab in recurrent small-cell lung cancer (CheckMate 032): A multicentre, open-label, phase 1/2 trial. *Lancet Oncol*, *17*(7), 883-895. https://doi.org/10.1016/s1470-2045(16)30098-5

Arasada, R. R., Amann, J. M., Rahman, M. A., Huppert, S. S., & Carbone, D. P. (2014). EGFR blockade enriches for lung cancer stem-like cells through Notch3-dependent signaling. *Cancer Res*, *74*(19), 5572-5584. https://doi.org/10.1158/0008-5472.Can-13-3724

Aschauer, L., & Muller, P. A. (2016). Novel targets and interaction partners of mutant p53 Gain-Of-Function. *Biochem Soc Trans*, *44*(2), 460-466. https://doi.org/10.1042/bst20150261

Aupérin, A., Le Péchoux, C., Rolland, E., Curran, W. J., Furuse, K., Fournel, P., Belderbos, J., Clamon, G., Ulutin, H. C., Paulus, R., Yamanaka, T., Bozonnat, M. C., Uitterhoeve, A., Wang, X., Stewart, L., Arriagada, R., Burdett, S., & Pignon, J. P. (2010). Meta-analysis of concomitant versus sequential radiochemotherapy in locally advanced non-small-cell lung cancer. *J Clin Oncol*, *28*(13), 2181-2190. https://doi.org/10.1200/jco.2009.26.2543

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., & Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*, *444*(7120), 756-760. https://doi.org/10.1038/nature05236

Bauer, M. K., Vogt, M., Los, M., Siegel, J., Wesselborg, S., & Schulze-Osthoff, K. (1998). Role of reactive oxygen intermediates in activation-induced CD95 (APO-1/Fas) ligand expression. *J Biol Chem*, *273*(14), 8048-8055. https://doi.org/10.1074/jbc.273.14.8048

Bayat Mokhtari, R., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., & Yeger, H. (2017). Combination therapy in combating cancer. *Oncotarget*, *8*(23), 38022-38043. https://doi.org/10.18632/oncotarget.16723

Bellamy, C. O. (1997). p53 and apoptosis. *Br Med Bull*, *53*(3), 522-538. https://doi.org/10.1093/oxfordjournals.bmb.a011628

Bergethon, K., Shaw, A. T., Ou, S. H., Katayama, R., Lovly, C. M., McDonald, N. T., Massion, P. P., Siwak-Tapp, C., Gonzalez, A., Fang, R., Mark, E. J., Batten, J. M., Chen, H., Wilner, K. D., Kwak, E. L., Clark, J. W., Carbone, D. P., Ji, H., Engelman, J. A., … Iafrate, A. J. (2012). ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol*, *30*(8), 863-870. https://doi.org/10.1200/jco.2011.35.6345

Bernier, J., Cooper, J. S., Pajak, T. F., van Glabbeke, M., Bourhis, J., Forastiere, A., Ozsahin, E. M., Jacobs, J. R., Jassem, J., Ang, K. K., & Lefèbvre, J. L. (2005). Defining risk levels in locally advanced head and neck cancers: a comparative analysis of concurrent postoperative radiation plus chemotherapy trials of the EORTC (#22931) and RTOG (# 9501). *Head Neck*, *27*(10), 843-850. https://doi.org/10.1002/hed.20279

Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefèbvre, J. L., Greiner, R. H., Giralt, J., Maingon, P., Rolland, F., Bolla, M., Cognetti, F., Bourhis, J., Kirkpatrick, A., & van Glabbeke, M. (2004). Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med*, *350*(19), 1945-1952. https://doi.org/10.1056/NEJMoa032641

Biscaro, A., Braga, A., & Berkowitz, R. S. (2015). Diagnosis, classification and treatment of gestational trophoblastic neoplasia. *Rev Bras Ginecol Obstet*, *37*(1), 42-51. https://doi.org/10.1590/so100-720320140005198

Blagosklonny, M. V. (2002). Sequential activation and inactivation of G2 checkpoints for selective killing of p53-deficient cells by microtubule-active drugs. *Oncogene*, *21*(41), 6249-6254. https://doi.org/10.1038/sj.onc.1205793

Board, P. D. Q. A. T. E. (2002). Small Cell Lung Cancer Treatment (PDQ®): Health Professional Version. In *PDQ Cancer Information Summaries*. National Cancer Institute (US).

Bonner, J. A., Harari, P. M., Giralt, J., Cohen, R. B., Jones, C. U., Sur, R. K., Raben, D., Baselga, J., Spencer, S. A., Zhu, J., Youssoufian, H., Rowinsky, E. K., & Ang, K. K. (2010). Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol*, *11*(1), 21-28. https://doi.org/10.1016/s1470-2045(09)70311-0

Burger, H., Nooter, K., Boersma, A. W., van Wingerden, K. E., Looijenga, L. H., Jochemsen, A. G., & Stoter, G. (1999). Distinct p53-independent apoptotic cell death signalling pathways in testicular germ cell tumour cell lines. *Int J Cancer*, *81*(4), 620-628. https://doi.org/10.1002/(sici)1097-0215(19990517)81:4<620::aid-ijc19>3.0.co;2-s

Burnette, W. N. (1981). "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem*, *112*(2), 195-203. https://doi.org/10.1016/0003-2697(81)90281-5

Califano, R., Abidin, A., Tariq, N. U., Economopoulou, P., Metro, G., & Mountzios, G. (2015). Beyond EGFR and ALK inhibition: Unravelling and exploiting novel genetic alterations in advanced non small-cell lung cancer. *Cancer Treat Rev*, *41*(5), 401-411. https://doi.org/10.1016/j.ctrv.2015.03.009

Castrogiovanni, C., Waterschoot, B., De Backer, O., & Dumont, P. (2018). Serine 392 phosphorylation modulates p53 mitochondrial translocation and transcription-

independent apoptosis. *Cell Death Differ*, *25*(1), 190-203. https://doi.org/10.1038/cdd.2017.143

CDC. (2019, 3-1-2021). *Mortality in the United States, 2019*. Retrieved 4-7-2021 from https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm

Chanes, R. E., Condit, P. T., Bottomley, R. H., & Nisimblat, W. (1971). Combined actinomycin D and vincristine in the treatment of patients with cancer. *Cancer*, *27*(3), 613-617. https://doi.org/10.1002/1097-0142(197103)27:3<613::aid-cncr2820270316>3.0.co;2-w

Charles, N., Ozawa, T., Squatrito, M., Bleau, A. M., Brennan, C. W., Hambardzumyan, D., & Holland, E. C. (2010). Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. *Cell Stem Cell*, *6*(2), 141-152. https://doi.org/10.1016/j.stem.2010.01.001

Chatziandreou, I., Tsioli, P., Sakellariou, S., Mourkioti, I., Giannopoulou, I., Levidou, G., Korkolopoulou, P., Patsouris, E., & Saetta, A. A. (2015). Comprehensive Molecular Analysis of NSCLC; Clinicopathological Associations. *PLoS One*, *10*(7), e0133859. https://doi.org/10.1371/journal.pone.0133859

Chen, W. J., Ho, C. C., Chang, Y. L., Chen, H. Y., Lin, C. A., Ling, T. Y., Yu, S. L., Yuan, S. S., Chen, Y. J., Lin, C. Y., Pan, S. H., Chou, H. Y., Chen, Y. J., Chang, G. C., Chu, W. C., Lee, Y. M., Lee, J. Y., Lee, P. J., Li, K. C., ... Yang, P. C. (2014). Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling. *Nat Commun*, *5*, 3472. https://doi.org/10.1038/ncomms4472

Cheok, C. F., & Lane, D. P. (2017). Exploiting the p53 Pathway for Therapy. *Cold Spring Harb Perspect Med*, *7*(3). https://doi.org/10.1101/cshperspect.a026310

Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D., & Green, D. R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science*, *309*(5741), 1732-1735. https://doi.org/10.1126/science.1114297

Choong, M. L., Yang, H., Lee, M. A., & Lane, D. P. (2009). Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy. *Cell Cycle*, *8*(17), 2810-2818. https://doi.org/10.4161/cc.8.17.9503

Chu, S., McDonald, T., Lin, A., Chakraborty, S., Huang, Q., Snyder, D. S., & Bhatia, R. (2011). Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. *Blood*, *118*(20), 5565-5572. https://doi.org/10.1182/blood-2010-12-327437

Cohen, E. E. W., Soulières, D., Le Tourneau, C., Dinis, J., Licitra, L., Ahn, M. J., Soria, A., Machiels, J. P., Mach, N., Mehra, R., Burtness, B., Zhang, P., Cheng, J., Swaby, R. F., & Harrington, K. J. (2019). Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): A randomised, open-label, phase 3 study. *Lancet*, *393*(10167), 156-167. https://doi.org/10.1016/s0140-6736(18)31999-8

Collins, L. G., Haines, C., Perkel, R., & Enck, R. E. (2007). Lung cancer: Diagnosis and management. *Am Fam Physician*, *75*(1), 56-63.

Cooper, J. S., Pajak, T. F., Forastiere, A. A., Jacobs, J., Campbell, B. H., Saxman, S. B., Kish, J. A., Kim, H. E., Cmelak, A. J., Rotman, M., Machtay, M., Ensley, J. F., Chao, K. S., Schultz, C. J., Lee, N., & Fu, K. K. (2004). Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med*, *350*(19), 1937-1944. https://doi.org/10.1056/NEJMoa032646

Cortes, C. L., Veiga, S. R., Almacellas, E., Hernández-Losa, J., Ferreres, J. C., Kozma, S. C., Ambrosio, S., Thomas, G., & Tauler, A. (2016). Effect of low doses of actinomycin D on neuroblastoma cell lines. *Mol Cancer*, *15*, 1. https://doi.org/10.1186/s12943-015-0489-8

Cragg, G. M., & Newman, D. J. (2000). Antineoplastic agents from natural sources: Achievements and future directions. *Expert Opin Investig Drugs*, *9*(12), 2783-2797. https://doi.org/10.1517/13543784.9.12.2783

Cramer, J. D., Burtness, B., Le, Q. T., & Ferris, R. L. (2019). The changing therapeutic landscape of head and neck cancer. *Nature Reviews. Clinical Oncology*, *16*(11), 669-683. https://doi.org/http://dx.doi.org/10.1038/s41571-019-0227-z

Creamer, M. R., Wang, T. W., Babb, S., Cullen, K. A., Day, H., Willis, G., Jamal, A., & Neff, L. (2019). Tobacco Product Use and Cessation Indicators Among Adults - United States, 2018. *MMWR Morb Mortal Wkly Rep*, *68*(45), 1013-1019. https://doi.org/10.15585/mmwr.mm6845a2

Crowley, L. C., Marfell, B. J., Scott, A. P., & Waterhouse, N. J. (2016). Quantitation of Apoptosis and Necrosis by Annexin V Binding, Propidium Iodide Uptake, and Flow Cytometry. *Cold Spring Harb Protoc*, *2016*(11). https://doi.org/10.1101/pdb.prot087288

Curran, W. J., Jr., Paulus, R., Langer, C. J., Komaki, R., Lee, J. S., Hauser, S., Movsas, B., Wasserman, T., Rosenthal, S. A., Gore, E., Machtay, M., Sause, W., & Cox, J. D. (2011). Sequential vs. concurrent chemoradiation for stage III non-small cell lung cancer: Randomized phase III trial RTOG 9410. *J Natl Cancer Inst*, *103*(19), 1452-1460. https://doi.org/10.1093/jnci/djr325

Dankner, M., Rose, A. A. N., Rajkumar, S., Siegel, P. M., & Watson, I. R. (2018). Classifying BRAF alterations in cancer: New rational therapeutic strategies for actionable mutations. *Oncogene*, *37*(24), 3183-3199. https://doi.org/10.1038/s41388-018-0171-x

Dearden, S., Stevens, J., Wu, Y. L., & Blowers, D. (2013). Mutation incidence and coincidence in non small-cell lung cancer: Meta-analyses by ethnicity and histology (mutMap). *Ann Oncol*, *24*(9), 2371-2376. https://doi.org/10.1093/annonc/mdt205

Decker, P., & Muller, S. (2002). Modulating poly (ADP-ribose) polymerase activity: Potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Curr Pharm Biotechnol*, *3*(3), 275-283. https://doi.org/10.2174/1389201023378265

Demain, A. L., & Vaishnav, P. (2011). Natural products for cancer chemotherapy. *Microb Biotechnol*, *4*(6), 687-699. https://doi.org/10.1111/j.1751-7915.2010.00221.x

Deneberg, S., Cherif, H., Lazarevic, V., Andersson, P. O., von Euler, M., Juliusson, G., & Lehmann, S. (2016). An open-label phase I dose-finding study of APR-246 in hematological malignancies. *Blood Cancer J*, *6*(7), e447. https://doi.org/10.1038/bcj.2016.60

Deng, X., Gao, F., Flagg, T., Anderson, J., & May, W. S. (2006). Bcl2's flexible loop domain regulates p53 binding and survival. *Mol Cell Biol*, *26*(12), 4421-4434. https://doi.org/10.1128/mcb.01647-05

Denis, F., Garaud, P., Bardet, E., Alfonsi, M., Sire, C., Germain, T., Bergerot, P., Rhein, B., Tortochaux, J., & Calais, G. (2004). Final results of the 94-01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy alone with

concomitant radiochemotherapy in advanced-stage oropharynx carcinoma. *J Clin Oncol*, *22*(1), 69-76. https://doi.org/10.1200/jco.2004.08.021

Devarakonda, S., Morgensztern, D., & Govindan, R. (2015). Genomic alterations in lung adenocarcinoma. *Lancet Oncol*, *16*(7), e342-351. https://doi.org/10.1016/s1470-2045(15)00077-7

Di Como, C. J., Gaiddon, C., & Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. *Mol Cell Biol*, *19*(2), 1438-1449. https://doi.org/10.1128/mcb.19.2.1438

El Dika, M. (2020). Redirecting E2F1 to TA-p73 improves cancer therapy through apoptotic induction. *DNA Repair (Amst)*, *90*, 102858. https://doi.org/10.1016/j.dnarep.2020.102858

Espinosa, J. M., Verdun, R. E., & Emerson, B. M. (2003). p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. *Mol Cell*, *12*(4), 1015-1027. https://doi.org/10.1016/s1097-2765(03)00359-9

Faber, A., Barth, C., Hörmann, K., Kassner, S., Schultz, J. D., Sommer, U., Stern-Straeter, J., Thorn, C., & Goessler, U. R. (2011). CD44 as a stem cell marker in head and neck squamous cell carcinoma. *Oncol Rep*, *26*(2), 321-326. https://doi.org/10.3892/or.2011.1322

Faber, A., Barth, C., Hörmann, K., Kassner, S., Schultz, J. D., Sommer, U., Stern-Straeter, J., Thorn, C., & Goessler, U. R. (2011). CD44 as a stem cell marker in head and neck squamous cell carcinoma. *Oncol Rep*, *26*(2), 321-326. https://doi.org/10.3892/or.2011.1322

Fakhry, C., Westra, W. H., Li, S., Cmelak, A., Ridge, J. A., Pinto, H., Forastiere, A., & Gillison, M. L. (2008). Improved Survival of Patients With Human Papillomavirus–Positive Head and Neck Squamous Cell Carcinoma in a Prospective Clinical Trial. *JNCI: Journal of the National Cancer Institute*, *100*(4), 261-269. https://doi.org/10.1093/jnci/djn011

Farber, S., D'Angio, G., Evans, A., & Mitus, A. (1960). Clinical studies on actinomycin D with special reference to Wilms' tumor in children. *Ann N Y Acad Sci*, *89*, 421-425. https://doi.org/10.1111/j.1749-6632.1960.tb20165.x

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2019). Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer*, *144*(8), 1941-1953. https://doi.org/10.1002/ijc.31937

Ferris, R. L., Blumenschein, G., Fayette, J., Guigay, J., Colevas, A. D., Licitra, L., Harrington, K., Kasper, S., Vokes, E. E., Even, C., Worden, F., Saba, N. F., Iglesias Docampo, L. C., Haddad, R., Rordorf, T., Kiyota, N., Tahara, M., Monga, M., ... Gillison, M. L. (2016). Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *New England Journal of Medicine*, *375*(19), 1856-1867. https://doi.org/10.1056/NEJMoa1602252

Ferris, R. L., Blumenschein, G., Jr., Fayette, J., Guigay, J., Colevas, A. D., Licitra, L., Harrington, K. J., Kasper, S., Vokes, E. E., Even, C., Worden, F., Saba, N. F., Docampo, L. C. I., Haddad, R., Rordorf, T., Kiyota, N., Tahara, M., Lynch, M., Jayaprakash, V., ... Gillison, M. L. (2018). Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of

CheckMate 141 with analyses by tumor PD-L1 expression. *Oral Oncol*, *81*, 45-51. https://doi.org/10.1016/j.oraloncology.2018.04.008

Finlay, C. A., Hinds, P. W., & Levine, A. J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell*, *57*(7), 1083-1093. https://doi.org/10.1016/0092-8674(89)90045-7

Fisher, M. J., Virmani, A. K., Wu, L., Aplenc, R., Harper, J. C., Powell, S. M., Rebbeck, T. R., Sidransky, D., Gazdar, A. F., & El-Deiry, W. S. (2001). Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer. *Clin Cancer Res*, *7*(6), 1688-1697.

Fontoura, B. M., Atienza, C. A., Sorokina, E. A., Morimoto, T., & Carroll, R. B. (1997). Cytoplasmic p53 polypeptide is associated with ribosomes. *Mol Cell Biol*, *17*(6), 3146-3154. https://doi.org/10.1128/mcb.17.6.3146

Forbes, S. A., Bindal, N., Bamford, S., Cole, C., Kok, C. Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A., Teague, J. W., Campbell, P. J., Stratton, M. R., & Futreal, P. A. (2011). COSMIC: Mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res*, *39*(Database issue), D945-950. https://doi.org/10.1093/nar/gkq929

Freed-Pastor, W. A., & Prives, C. (2012). Mutant p53: One name, many proteins. *Genes Dev*, *26*(12), 1268-1286. https://doi.org/10.1101/gad.190678.112

Frei, E., 3rd, Karon, M., Levin, R. H., Freireich, E. J., Taylor, R. J., Hananian, J., Selawry, O., Holland, J. F., Hoogstraten, B., Wolman, I. J., Abir, E., Sawitsky, A., Lee, S., Mills, S. D., Burgert, E. O., Jr., Spurr, C. L., Patterson, R. B., Ebaugh, F. G., James, G. W., 3rd, &

Moon, J. H. (1965). The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia. *Blood*, *26*(5), 642-656.

Fridman, J. S., & Lowe, S. W. (2003). Control of apoptosis by p53. *Oncogene*, *22*(56), 9030-9040. https://doi.org/10.1038/sj.onc.1207116

Fried, D. B., Morris, D. E., Poole, C., Rosenman, J. G., Halle, J. S., Detterbeck, F. C., Hensing, T. A., & Socinski, M. A. (2004). Systematic review evaluating the timing of thoracic radiation therapy in combined modality therapy for limited-stage small-cell lung cancer. *J Clin Oncol*, *22*(23), 4837-4845. https://doi.org/10.1200/jco.2004.01.178

Friesen, C., Fulda, S., & Debatin, K. M. (1997). Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. *Leukemia*, *11*(11), 1833-1841. https://doi.org/10.1038/sj.leu.2400827

Fujimoto, J., & Wistuba, II. (2014). Current concepts on the molecular pathology of non-small cell lung carcinoma. *Semin Diagn Pathol*, *31*(4), 306-313. https://doi.org/10.1053/j.semdp.2014.06.008

Fulda, S. (2009). Tumor resistance to apoptosis. *Int J Cancer*, *124*(3), 511-515. https://doi.org/10.1002/ijc.24064

Fulda, S., Los, M., Friesen, C., & Debatin, K. M. (1998). Chemosensitivity of solid tumor cells in vitro is related to activation of the CD95 system. *Int J Cancer*, *76*(1), 105-114. https://doi.org/10.1002/(sici)1097-0215(19980330)76:1<105::aid-ijc17>3.0.co;2-b

Gainor, J. F., Varghese, A. M., Ou, S. H., Kabraji, S., Awad, M. M., Katayama, R., Pawlak, A., Mino-Kenudson, M., Yeap, B. Y., Riely, G. J., Iafrate, A. J., Arcila, M. E., Ladanyi, M., Engelman, J. A., Dias-Santagata, D., & Shaw, A. T. (2013). ALK rearrangements are mutually exclusive with mutations in EGFR or KRAS: An analysis of 1,683 patients with

non-small cell lung cancer. *Clin Cancer Res*, *19*(15), 4273-4281. https://doi.org/10.1158/1078-0432.Ccr-13-0318

Gaspar, N., Hawkins, D. S., Dirksen, U., Lewis, I. J., Ferrari, S., Le Deley, M. C., Kovar, H., Grimer, R., Whelan, J., Claude, L., Delattre, O., Paulussen, M., Picci, P., Sundby Hall, K., van den Berg, H., Ladenstein, R., Michon, J., Hjorth, L., Judson, I., ... Oberlin, O. (2015). Ewing Sarcoma: Current Management and Future Approaches Through Collaboration. *J Clin Oncol*, *33*(27), 3036-3046. https://doi.org/10.1200/jco.2014.59.5256

Geoffrois, L., Martin, L., De Raucourt, D., Sun, X. S., Tao, Y., Maingon, P., Buffet, J., Pointreau, Y., Sire, C., Tuchais, C., Babin, E., Coutte, A., Rolland, F., Kaminsky, M. C., Alfonsi, M., Lapeyre, M., Saliou, M., Lafond, C., Jadaud, E., ... Bourhis, J. (2018). Induction Chemotherapy Followed by Cetuximab Radiotherapy Is Not Superior to Concurrent Chemoradiotherapy for Head and Neck Carcinomas: Results of the GORTEC 2007-02 Phase III Randomized Trial. *J Clin Oncol*, *36*(31), 3077-3083. https://doi.org/10.1200/jco.2017.76.2591

Gillison, M. L., Trotti, A. M., Harris, J., Eisbruch, A., Harari, P. M., Adelstein, D. J., Jordan, R. C. K., Zhao, W., Sturgis, E. M., Burtness, B., Ridge, J. A., Ringash, J., Galvin, J., Yao, M., Koyfman, S. A., Blakaj, D. M., Razaq, M. A., Colevas, A. D., Beitler, J. J., ... Le, Q. T. (2019). Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): A randomised, multicentre, non-inferiority trial. *Lancet*, *393*(10166), 40-50. https://doi.org/10.1016/s0140-6736(18)32779-x

Globocan. (2020). *Cancer Fact Sheet*. https://gco.iarc.fr/today/fact-sheets-cancers

Goh, A. M., Coffill, C. R., & Lane, D. P. (2011). The role of mutant p53 in human cancer. *J Pathol*, *223*(2), 116-126. https://doi.org/10.1002/path.2784

Goldberg, I. H., & Rabinowitz, M. (1962). Actinomycin D Inhibition of Deoxyribonucleic Acid-Dependent Synthesis of Ribonucleic Acid. *Science*, *136*(3513), 315-316. https://doi.org/10.1126/science.136.3513.315

Goldstraw, P., Chansky, K., Crowley, J., Rami-Porta, R., Asamura, H., Eberhardt, W. E., Nicholson, A. G., Groome, P., Mitchell, A., & Bolejack, V. (2016). The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer. *J Thorac Oncol*, *11*(1), 39-51. https://doi.org/10.1016/j.jtho.2015.09.009

Gonzalvez, F., & Ashkenazi, A. (2010). New insights into apoptosis signaling by Apo2L/TRAIL. *Oncogene*, *29*(34), 4752-4765. https://doi.org/10.1038/onc.2010.221

Govindan, R., Szczesna, A., Ahn, M. J., Schneider, C. P., Gonzalez Mella, P. F., Barlesi, F., Han, B., Ganea, D. E., Von Pawel, J., Vladimirov, V., Fadeeva, N., Lee, K. H., Kurata, T., Zhang, L., Tamura, T., Postmus, P. E., Jassem, J., O'Byrne, K., Kopit, J., ... Reck, M. (2017). Phase III Trial of Ipilimumab Combined With Paclitaxel and Carboplatin in Advanced Squamous Non-Small-Cell Lung Cancer. *J Clin Oncol*, *35*(30), 3449-3457. https://doi.org/10.1200/jco.2016.71.7629

Green, D. R. (2000). Apoptotic pathways: Paper wraps stone blunts scissors. *Cell*, *102*(1), 1-4. https://doi.org/10.1016/s0092-8674(00)00003-9

Green, R., Howell, M., Khalil, R., Nair, R., Yan, J., Foran, E., Katiri, S., Banerjee, J., Singh, M., Bharadwaj, S., Mohapatra, S. S., & Mohapatra, S. (2019). Actinomycin D and

Telmisartan Combination Targets Lung Cancer Stem Cells Through the Wnt/Beta Catenin Pathway. *Sci Rep*, *9*(1), 18177. https://doi.org/10.1038/s41598-019-54266-z

Griffon, G., Merlin, J. L., & Marchal, C. (1995). Comparison of sulforhodamine B, tetrazolium and clonogenic assays for in vitro radiosensitivity testing in human ovarian cell lines. *Anticancer Drugs*, *6*(1), 115-123. https://doi.org/10.1097/00001813-199502000-00014

Guo, L., Fan, L., Ren, J., Pang, Z., Ren, Y., Li, J., Wen, Z., Qian, Y., Zhang, L., Ma, H., & Jiang, X. (2012). Combination of TRAIL and actinomycin D liposomes enhances antitumor effect in non-small cell lung cancer. *Int J Nanomedicine*, *7*, 1449-1460. https://doi.org/10.2147/ijn.S24711

Hammerman, P. S., Lawrence, M. S., Voet, D., Jing, R., Cibulskis, K., Sivachenko, A., Stojanov, P., McKenna, A., Lander, E. S., Gabriel, S., Getz, G., Sougnez, C., Imielinski, M., Helman, E., Hernandez, B., Pho, N. H., Meyerson, M., Chu, A., Chun, H.-J. E., ... Pao, W. (2012). Comprehensive genomic characterization of squamous cell lung cancers. *Nature*, *489*(7417), 519-525. https://doi.org/10.1038/nature11404

Han, Y., Liu, D., & Li, L. (2020). PD-1/PD-L1 pathway: current researches in cancer. *Am J Cancer Res*, *10*(3), 727-742.

Hann, C. L., & Rudin, C. M. (2008). Management of small-cell lung cancer: Incremental changes but hope for the future. *Oncology (Williston Park)*, *22*(13), 1486-1492.

Harrington, K. J., Ferris, R. L., Blumenschein, G., Jr., Colevas, A. D., Fayette, J., Licitra, L., Kasper, S., Even, C., Vokes, E. E., Worden, F., Saba, N. F., Kiyota, N., Haddad, R., Tahara, M., Grünwald, V., Shaw, J. W., Monga, M., Lynch, M., Taylor, F., ... Guigay, J. (2017). Nivolumab versus standard, single-agent therapy of investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck (CheckMate 141):

Health-related quality-of-life results from a randomised, phase 3 trial. *Lancet Oncol*, *18*(8), 1104-1115. https://doi.org/10.1016/s1470-2045(17)30421-7

Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M. P., Maso, L. D., Daudt, A. W., Fabianova, E., Wünsch-Filho, V., Franceschi, S., Hayes, R. B., Herrero, R., Koifman, S., La Vecchia, C., Lazarus, P., Levi, F., Mates, D., Matos, E., ... Boffetta, P. (2007). 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. *JNCI: Journal of the National Cancer Institute*, *99*(10), 777-789. https://doi.org/10.1093/jnci/djk179

Health, N. C. f. C. D. P. a. H. P. U. O. o. S. a. (1964). *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*. https://www.ncbi.nlm.nih.gov/books/NBK294311/

Hirsch, F. R., Scagliotti, G. V., Mulshine, J. L., Kwon, R., Curran, W. J., Wu, Y.-L., & Paz-Ares, L. (2017). Lung cancer: Current therapies and new targeted treatments. *The Lancet*, *389*(10066), 299-311. https://doi.org/https://doi.org/10.1016/S0140-6736(16)30958-8

Ho, A. L., Chau, N. G., Wong, D. J. L., Cabanillas, M. E., Bauman, J. R., Bible, K. C., Brose, M. S., Calvo, E., Boni, V., Burrows, F., Melvin, C. L., Scholz, C. R., & Gualberto, A. (2017). An open-label, phase II study of tipifarnib for the treatment of HRAS mutant solid tumors, including squamous cell carcinomas of the head and neck. *Journal of Clinical Oncology*, *35*(15_suppl), TPS2618-TPS2618. https://doi.org/10.1200/JCO.2017.35.15_suppl.TPS2618

Ho, T., Tan, B. X., & Lane, D. (2019). How the Other Half Lives: What p53 Does When It Is Not Being a Transcription Factor. *Int J Mol Sci*, *21*(1). https://doi.org/10.3390/ijms21010013

Hollstein, U. (1974). Actinomycin. Chemistry and mechanism of action. *Chemical Reviews*, *74*(6), 625-652. https://doi.org/10.1021/cr60292a002

Hopkins-Donaldson, S., Ziegler, A., Kurtz, S., Bigosch, C., Kandioler, D., Ludwig, C., Zangemeister-Wittke, U., & Stahel, R. (2003). Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. *Cell Death Differ*, *10*(3), 356-364. https://doi.org/10.1038/sj.cdd.4401157

Horn, L., Mansfield, A. S., Szczęsna, A., Havel, L., Krzakowski, M., Hochmair, M. J., Huemer, F., Losonczy, G., Johnson, M. L., Nishio, M., Reck, M., Mok, T., Lam, S., Shames, D. S., Liu, J., Ding, B., Lopez-Chavez, A., Kabbinavar, F., Lin, W., ... Liu, S. V. (2018). First-Line Atezolizumab plus Chemotherapy in Extensive-Stage Small-Cell Lung Cancer. *N Engl J Med*, *379*(23), 2220-2229. https://doi.org/10.1056/NEJMoa1809064

Hosoi, H. (2016). Current status of treatment for pediatric rhabdomyosarcoma in the USA and Japan. *Pediatr Int*, *58*(2), 81-87. https://doi.org/10.1111/ped.12867

Hotta, K., Aoe, K., Kozuki, T., Ohashi, K., Ninomiya, K., Ichihara, E., Kubo, T., Ninomiya, T., Chikamori, K., Harada, D., Nogami, N., Hirata, T., Hinotsu, S., Toyooka, S., & Kiura, K. (2018). A Phase II Study of Trastuzumab Emtansine in HER2-Positive Non-Small Cell Lung Cancer. *J Thorac Oncol*, *13*(2), 273-279. https://doi.org/10.1016/j.jtho.2017.10.032

Huang, S. F., Liu, H. P., Li, L. H., Ku, Y. C., Fu, Y. N., Tsai, H. Y., Chen, Y. T., Lin, Y. F., Chang, W. C., Kuo, H. P., Wu, Y. C., Chen, Y. R., & Tsai, S. F. (2004). High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell

lung cancers related to gefitinib responsiveness in Taiwan. *Clin Cancer Res*, *10*(24), 8195-8203. https://doi.org/10.1158/1078-0432.Ccr-04-1245

Hurwitz, J., Furth, J. J., Malamy, M., & Alexander, M. (1962). The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavin. *Proc Natl Acad Sci U S A*, *48*(7), 1222-1230. https://doi.org/10.1073/pnas.48.7.1222

Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. S., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden, K. H., & Kaelin, W. G., Jr. (2000). Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature*, *407*(6804), 645-648. https://doi.org/10.1038/35036614

Jänne, P. A., Shaw, A. T., Pereira, J. R., Jeannin, G., Vansteenkiste, J., Barrios, C., Franke, F. A., Grinsted, L., Zazulina, V., Smith, P., Smith, I., & Crinò, L. (2013). Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: A randomised, multicentre, placebo-controlled, phase 2 study. *Lancet Oncol*, *14*(1), 38-47. https://doi.org/10.1016/s1470-2045(12)70489-8

Jensen, E. C. (2012). The basics of western blotting. *Anat Rec (Hoboken)*, *295*(3), 369-371. https://doi.org/10.1002/ar.22424

Joerger, A. C., & Fersht, A. R. (2016). The p53 Pathway: Origins, Inactivation in Cancer, and Emerging Therapeutic Approaches. *Annu Rev Biochem*, *85*, 375-404. https://doi.org/10.1146/annurev-biochem-060815-014710

Johnson, D. E., Burtness, B., Leemans, C. R., Lui, V. W. Y., Bauman, J. E., & Grandis, J. R. (2020). Head and neck squamous cell carcinoma. *Nature Reviews Disease Primers*, *6*(1), 92. https://doi.org/10.1038/s41572-020-00224-3

Jung, K., Kang, H., & Mehra, R. (2018). Targeting phosphoinositide 3-kinase (PI3K) in head and neck squamous cell carcinoma (HNSCC). *Cancers of the Head & Neck*, *3*(1), 3. https://doi.org/10.1186/s41199-018-0030-z

Kalemkerian, G. P., Akerley, W., Bogner, P., Borghaei, H., Chow, L. Q., Downey, R. J., Gandhi, L., Ganti, A. K., Govindan, R., Grecula, J. C., Hayman, J., Heist, R. S., Horn, L., Jahan, T., Koczywas, M., Loo, B. W., Jr., Merritt, R. E., Moran, C. A., Niell, H. B., ... Hughes, M. (2013). Small cell lung cancer. *J Natl Compr Canc Netw*, *11*(1), 78-98. https://doi.org/10.6004/jnccn.2013.0011

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, *51*(23 Pt 1), 6304-6311.

Kastenhuber, E. R., & Lowe, S. W. (2017). Putting p53 in Context. *Cell*, *170*(6), 1062-1078. https://doi.org/10.1016/j.cell.2017.08.028

Kazandjian, D., Blumenthal, G. M., Chen, H. Y., He, K., Patel, M., Justice, R., Keegan, P., & Pazdur, R. (2014). FDA approval summary: Crizotinib for the treatment of metastatic non-small cell lung cancer with anaplastic lymphoma kinase rearrangements. *Oncologist*, *19*(10), e5-11. https://doi.org/10.1634/theoncologist.2014-0241

Keepers, Y. P., Pizao, P. E., Peters, G. J., van Ark-Otte, J., Winograd, B., & Pinedo, H. M. (1991). Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. *Eur J Cancer*, *27*(7), 897-900. https://doi.org/10.1016/0277-5379(91)90142-z

Khazir, J., Riley, D. L., Pilcher, L. A., De-Maayer, P., & Mir, B. A. (2014). Anticancer agents from diverse natural sources. *Nat Prod Commun*, *9*(11), 1655-1669.

Kimura, S. (2016). Current status of ABL tyrosine kinase inhibitors stop studies for chronic myeloid leukemia. *Stem Cell Investig*, *3*, 36. https://doi.org/10.21037/sci.2016.07.08

Kleeff, J., Kornmann, M., Sawhney, H., & Korc, M. (2000). Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells. *Int J Cancer*, *86*(3), 399-407. https://doi.org/10.1002/(sici)1097-0215(20000501)86:3<399::aid-ijc15>3.0.co;2-g

Koba, M., & Konopa, J. (2005). [Actinomycin D and its mechanisms of action]. *Postepy Hig Med Dosw (Online)*, *59*, 290-298. (Aktynomycyna D i mechanizmy jej działania.)

Kosaka, T., Yatabe, Y., Endoh, H., Kuwano, H., Takahashi, T., & Mitsudomi, T. (2004). Mutations of the epidermal growth factor receptor gene in lung cancer: Biological and clinical implications. *Cancer Res*, *64*(24), 8919-8923. https://doi.org/10.1158/0008-5472.Can-04-2818

Kumari, R., Kohli, S., & Das, S. (2014). p53 regulation upon genotoxic stress: Intricacies and complexities. *Mol Cell Oncol*, *1*(3), e969653. https://doi.org/10.4161/23723548.2014.969653

Kurien, B. T., & Scofield, R. H. (2006). Western blotting. *Methods*, *38*(4), 283-293. https://doi.org/10.1016/j.ymeth.2005.11.007

Lalami, Y., & Awada, A. (2016). Innovative perspectives of immunotherapy in head and neck cancer. From relevant scientific rationale to effective clinical practice. *Cancer Treat Rev*, *43*, 113-123. https://doi.org/10.1016/j.ctrv.2016.01.001

Lara, P. N., Jr., Natale, R., Crowley, J., Lenz, H. J., Redman, M. W., Carleton, J. E., Jett, J., Langer, C. J., Kuebler, J. P., Dakhil, S. R., Chansky, K., & Gandara, D. R. (2009). Phase III trial of irinotecan/cisplatin compared with etoposide/cisplatin in extensive-stage

small-cell lung cancer: Clinical and pharmacogenomic results from SWOG S0124. *J Clin Oncol*, *27*(15), 2530-2535. https://doi.org/10.1200/jco.2008.20.1061

Lawrence, M. S., Sougnez, C., Lichtenstein, L., Cibulskis, K., Lander, E., Gabriel, S. B., Getz, G., Ally, A., Balasundaram, M., Birol, I., Bowlby, R., Brooks, D., Butterfield, Y. S. N., Carlsen, R., Cheng, D., Chu, A., Dhalla, N., Guin, R., Holt, R. A., … Burnett, K. (2015). Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*, *517*(7536), 576-582. https://doi.org/10.1038/nature14129

Lawrie, T. A., Alazzam, M., Tidy, J., Hancock, B. W., & Osborne, R. (2016). First-line chemotherapy in low-risk gestational trophoblastic neoplasia. *Cochrane Database Syst Rev*, *2016*(6), Cd007102. https://doi.org/10.1002/14651858.CD007102.pub4

Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Jang, J. J., Han, J. Y., Lee, J. Y., & Yoo, N. J. (1999). Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. *Cancer Res*, *59*(22), 5683-5686.

Leroy, B., Anderson, M., & Soussi, T. (2014). TP53 mutations in human cancer: Database reassessment and prospects for the next decade. *Hum Mutat*, *35*(6), 672-688. https://doi.org/10.1002/humu.22552

Liu, G., Pei, F., Yang, F., Li, L., Amin, A. D., Liu, S., Buchan, J. R., & Cho, W. C. (2017). Role of Autophagy and Apoptosis in Non-Small-Cell Lung Cancer. *Int J Mol Sci*, *18*(2). https://doi.org/10.3390/ijms18020367

Liu, X., Yue, P., Khuri, F. R., & Sun, S. Y. (2004). p53 upregulates death receptor 4 expression through an intronic p53 binding site. *Cancer Res*, *64*(15), 5078-5083. https://doi.org/10.1158/0008-5472.Can-04-1195

Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., & Tlsty, T. D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, *70*(6), 923-935. https://doi.org/10.1016/0092-8674(92)90243-6

Lynch, T. J., Bondarenko, I., Luft, A., Serwatowski, P., Barlesi, F., Chacko, R., Sebastian, M., Neal, J., Lu, H., Cuillerot, J. M., & Reck, M. (2012). Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. *J Clin Oncol*, *30*(17), 2046-2054. https://doi.org/10.1200/jco.2011.38.4032

Lytle, N. K., Barber, A. G., & Reya, T. (2018). Stem cell fate in cancer growth, progression and therapy resistance. *Nat Rev Cancer*, *18*(11), 669-680. https://doi.org/10.1038/s41568-018-0056-x

Malhotra, J., Malvezzi, M., Negri, E., La Vecchia, C., & Boffetta, P. (2016). Risk factors for lung cancer worldwide. *Eur Respir J*, *48*(3), 889-902. https://doi.org/10.1183/13993003.00359-2016

Malta, T. M., Sokolov, A., Gentles, A. J., Burzykowski, T., Poisson, L., Weinstein, J. N., Kamińska, B., Huelsken, J., Omberg, L., Gevaert, O., Colaprico, A., Czerwińska, P., Mazurek, S., Mishra, L., Heyn, H., Krasnitz, A., Godwin, A. K., Lazar, A. J., Stuart, J. M., ...Wiznerowicz, M. (2018). Machine Learning Identifies Stemness Features Associated with Oncogenic Dedifferentiation. *Cell*, *173*(2), 338-354.e315. https://doi.org/10.1016/j.cell.2018.03.034

Mandal, R., Şenbabaoğlu, Y., Desrichard, A., Havel, J. J., Dalin, M. G., Riaz, N., Lee, K. W., Ganly, I., Hakimi, A. A., Chan, T. A., & Morris, L. G. (2016). The head and neck cancer

immune landscape and its immunotherapeutic implications. *JCI Insight*, *1*(17), e89829. https://doi.org/10.1172/jci.insight.89829

Mandruzzato, S., Brasseur, F., Andry, G., Boon, T., & van der Bruggen, P. (1997). A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. *J Exp Med*, *186*(5), 785-793. https://doi.org/10.1084/jem.186.5.785

Mao, Y., Yang, D., He, J., & Krasna, M. J. (2016). Epidemiology of Lung Cancer. *Surg Oncol Clin N Am*, *25*(3), 439-445. https://doi.org/10.1016/j.soc.2016.02.001

Markham, A. (2020). Selpercatinib: First Approval. *Drugs*, *80*(11), 1119-1124. https://doi.org/10.1007/s40265-020-01343-7

Mazieres, J., Gandara, S. R., Leighl, N., Wheler, J. J., Barlesi, F., & Zalcman, G. (2013). Oral MEK1/MEK2 inhibitor trametinib (GSK1120212) in combination with pemetrexed in a phase 1/1B trial involving KRAS-mutant and wild-type (WT) advanced non-small cell lung cancer (NSCLC): Efficacy and biomarker results. *J Thorac Oncol*, *8*.

Mazières, J., Peters, S., Lepage, B., Cortot, A. B., Barlesi, F., Beau-Faller, M., Besse, B., Blons, H., Mansuet-Lupo, A., Urban, T., Moro-Sibilot, D., Dansin, E., Chouaid, C., Wislez, M., Diebold, J., Felip, E., Rouquette, I., Milia, J. D., & Gautschi, O. (2013). Lung cancer that harbors an HER2 mutation: Epidemiologic characteristics and therapeutic perspectives. *J Clin Oncol*, *31*(16), 1997-2003. https://doi.org/10.1200/jco.2012.45.6095

McKenzie, P. P., McPake, C. R., Ashford, A. A., Vanin, E. F., & Harris, L. C. (2002). MDM2 does not influence p53-mediated sensitivity to DNA-damaging drugs. *Mol Cancer Ther*, *1*(12), 1097-1104.

Mehanna, H., Beech, T., Nicholson, T., El-Hariry, I., McConkey, C., Paleri, V., & Roberts, S. (2013). Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal

head and neck cancer--systematic review and meta-analysis of trends by time and region. *Head Neck*, *35*(5), 747-755. https://doi.org/10.1002/hed.22015

Mehanna, H., Robinson, M., Hartley, A., Kong, A., Foran, B., Fulton-Lieuw, T., Dalby, M., Mistry, P., Sen, M., O'Toole, L., Al Booz, H., Dyker, K., Moleron, R., Whitaker, S., Brennan, S., Cook, A., Griffin, M., Aynsley, E., Rolles, M., ... Dunn, J. (2019). Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. *Lancet*, *393*(10166), 51-60. https://doi.org/10.1016/s0140-6736(18)32752-1

Méndez-García, L. A., Martínez-Castillo, M., Villegas-Sepúlveda, N., Orozco, L., & Córdova, E. J. (2019). Curcumin induces p53-independent inactivation of Nrf2 during oxidative stress-induced apoptosis. *Hum Exp Toxicol*, *38*(8), 951-961. https://doi.org/10.1177/0960327119845035

Messina, R. L., Sanfilippo, M., Vella, V., Pandini, G., Vigneri, P., Nicolosi, M. L., Gianì, F., Vigneri, R., & Frasca, F. (2012). Reactivation of p53 mutants by prima-1 [corrected] in thyroid cancer cells. *Int J Cancer*, *130*(10), 2259-2270. https://doi.org/10.1002/ijc.26228

Molina-Vila, M. A., Bertran-Alamillo, J., Gascó, A., Mayo-de-las-Casas, C., Sánchez-Ronco, M., Pujantell-Pastor, L., Bonanno, L., Favaretto, A. G., Cardona, A. F., Vergnenègre, A., Majem, M., Massuti, B., Morán, T., Carcereny, E., Viteri, S., & Rosell, R. (2014). Nondisruptive p53 mutations are associated with shorter survival in patients with advanced non-small cell lung cancer. *Clin Cancer Res*, *20*(17), 4647-4659. https://doi.org/10.1158/1078-0432.Ccr-13-2391

Morachis, J. M., Murawsky, C. M., & Emerson, B. M. (2010). Regulation of the p53 transcriptional response by structurally diverse core promoters. *Genes Dev*, *24*(2), 135-147. https://doi.org/10.1101/gad.1856710

Moskovitz, J., Moy, J., & Ferris, R. L. (2018). Immunotherapy for Head and Neck Squamous Cell Carcinoma. *Curr Oncol Rep*, *20*(2), 22. https://doi.org/10.1007/s11912-018-0654-5

Muir, C., & Weiland, L. (1995). Upper aerodigestive tract cancers. *Cancer*, *75*(1 Suppl), 147-153. https://doi.org/10.1002/1097-0142(19950101)75:1+<147::aid-cncr2820751304>3.0.co;2-u

Muller, P. A., Trinidad, A. G., Timpson, P., Morton, J. P., Zanivan, S., van den Berghe, P. V., Nixon, C., Karim, S. A., Caswell, P. T., Noll, J. E., Coffill, C. R., Lane, D. P., Sansom, O. J., Neilsen, P. M., Norman, J. C., & Vousden, K. H. (2013). Mutant p53 enhances MET trafficking and signalling to drive cell scattering and invasion. *Oncogene*, *32*(10), 1252-1265. https://doi.org/10.1038/onc.2012.148

Mullis, K. B., & Faloona, F. A. (1987). [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In *Methods in Enzymology* (Vol. 155, pp. 335-350). Academic Press. https://doi.org/https://doi.org/10.1016/0076-6879(87)55023-6

Myers, T. W., & Gelfand, D. H. (1991). Reverse transcription and DNA amplification by a Thermus thermophilus DNA polymerase. *Biochemistry*, *30*(31), 7661-7666. https://doi.org/10.1021/bi00245a001

Näsman, A., Romanitan, M., Nordfors, C., Grün, N., Johansson, H., Hammarstedt, L., Marklund, L., Munck-Wikland, E., Dalianis, T., & Ramqvist, T. (2012). Tumor infiltrating CD8+ and Foxp3+ lymphocytes correlate to clinical outcome and human papillomavirus (HPV)

status in tonsillar cancer. *PLoS One*, *7*(6), e38711. https://doi.org/10.1371/journal.pone.0038711

NCI. (2021). *Cancer Stat Facts : Lung and Bronchus Cancer*. seer.cancer.gov/statfacts/html/lungb.html

Neal, R. D., Hamilton, W., & Rogers, T. K. (2014). Lung cancer. *Bmj*, *349*, g6560. https://doi.org/10.1136/bmj.g6560

Noda, K., Nishiwaki, Y., Kawahara, M., Negoro, S., Sugiura, T., Yokoyama, A., Fukuoka, M., Mori, K., Watanabe, K., Tamura, T., Yamamoto, S., & Saijo, N. (2002). Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med*, *346*(2), 85-91. https://doi.org/10.1056/NEJMoa003034

Nordfors, C., Grün, N., Tertipis, N., Ährlund-Richter, A., Haeggblom, L., Sivars, L., Du, J., Nyberg, T., Marklund, L., Munck-Wikland, E., Näsman, A., Ramqvist, T., & Dalianis, T. (2013). CD8+ and CD4+ tumour infiltrating lymphocytes in relation to human papillomavirus status and clinical outcome in tonsillar and base of tongue squamous cell carcinoma. *Eur J Cancer*, *49*(11), 2522-2530. https://doi.org/10.1016/j.ejca.2013.03.019

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., & Taya, Y. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell*, *102*(6), 849-862. https://doi.org/10.1016/s0092-8674(00)00073-8

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: Origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol*, *2*(1), a001008. https://doi.org/10.1101/cshperspect.a001008

Ong, D. S. T., Hu, B., Ho, Y. W., Sauvé, C. G., Bristow, C. A., Wang, Q., Multani, A. S., Chen, P., Nezi, L., Jiang, S., Gorman, C. E., Monasterio, M. M., Koul, D., Marchesini, M., Colla, S., Jin, E. J., Sulman, E. P., Spring, D. J., Yung, W. A., ... DePinho, R. A. (2017). PAF promotes stemness and radioresistance of glioma stem cells. *Proc Natl Acad Sci U S A*, *114*(43), E9086-e9095. https://doi.org/10.1073/pnas.1708122114

Oosting, S. F., & Haddad, R. I. (2019). Best Practice in Systemic Therapy for Head and Neck Squamous Cell Carcinoma [Review]. *Frontiers in Oncology*, *9*(815). https://doi.org/10.3389/fonc.2019.00815

Orellana, E. A., & Kasinski, A. L. (2016). Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. *Bio Protoc*, *6*(21). https://doi.org/10.21769/BioProtoc.1984

Osazuwa-Peters, N., Simpson, M. C., Zhao, L., Boakye, E. A., Olomukoro, S. I., Deshields, T., Loux, T. M., Varvares, M. A., & Schootman, M. (2018). Suicide risk among cancer survivors: Head and neck versus other cancers. *Cancer*, *124*(20), 4072-4079. https://doi.org/10.1002/cncr.31675

Ott, P. A., Elez, E., Hiret, S., Kim, D. W., Morosky, A., Saraf, S., Piperdi, B., & Mehnert, J. M. (2017). Pembrolizumab in Patients With Extensive-Stage Small-Cell Lung Cancer: Results From the Phase Ib KEYNOTE-028 Study. *J Clin Oncol*, *35*(34), 3823-3829. https://doi.org/10.1200/jco.2017.72.5069

Paik, P. K., Drilon, A., Fan, P. D., Yu, H., Rekhtman, N., Ginsberg, M. S., Borsu, L., Schultz, N., Berger, M. F., Rudin, C. M., & Ladanyi, M. (2015). Response to MET inhibitors in patients with stage IV lung adenocarcinomas harboring MET mutations causing exon 14 skipping. *Cancer Discov*, *5*(8), 842-849. https://doi.org/10.1158/2159-8290.Cd-14-1467

Parant, J. M., & Lozano, G. (2003). Disrupting TP53 in mouse models of human cancers. *Hum Mutat*, *21*(3), 321-326. https://doi.org/10.1002/humu.10186

Paz-Ares, L., Ciuleanu, T. E., Cobo, M., Schenker, M., Zurawski, B., Menezes, J., Richardet, E., Bennouna, J., Felip, E., Juan-Vidal, O., Alexandru, A., Sakai, H., Lingua, A., Salman, P., Souquet, P. J., De Marchi, P., Martin, C., Pérol, M., Scherpereel, A., ... Reck, M. (2021). First-line nivolumab plus ipilimumab combined with two cycles of chemotherapy in patients with non-small-cell lung cancer (CheckMate 9LA): An international, randomised, open-label, phase 3 trial. *Lancet Oncol*, *22*(2), 198-211. https://doi.org/10.1016/s1470-2045(20)30641-0

Paz-Ares, L., Dvorkin, M., Chen, Y., Reinmuth, N., Hotta, K., Trukhin, D., Statsenko, G., Hochmair, M. J., Özgüroğlu, M., Ji, J. H., Voitko, O., Poltoratskiy, A., Ponce, S., Verderame, F., Havel, L., Bondarenko, I., Kazarnowicz, A., Losonczy, G., Conev, N. V., ... Goldman, J. W. (2019). Durvalumab plus platinum-etoposide versus platinum-etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): A randomised, controlled, open-label, phase 3 trial. *Lancet*, *394*(10212), 1929-1939. https://doi.org/10.1016/s0140-6736(19)32222-6

Perry, R. P., & Kelley, D. E. (1970). Inhibition of RNA synthesis by actinomycin D: Characteristic dose-response of different RNA species. *J Cell Physiol*, *76*(2), 127-139. https://doi.org/10.1002/jcp.1040760202

Peters, S., Stahel, R., Bubendorf, L., Bonomi, P., Villegas, A., Kowalski, D. M., Baik, C. S., Isla, D., Carpeno, J. C., Garrido, P., Rittmeyer, A., Tiseo, M., Meyenberg, C., de Haas, S., Lam, L. H., Lu, M. W., & Stinchcombe, T. E. (2019). Trastuzumab Emtansine (T-DM1) in Patients with Previously Treated HER2-Overexpressing Metastatic Non-Small Cell

Lung Cancer: Efficacy, Safety, and Biomarkers. *Clin Cancer Res*, *25*(1), 64-72. https://doi.org/10.1158/1078-0432.Ccr-18-1590

Pfister, N. T., & Prives, C. (2017). Transcriptional Regulation by Wild-Type and Cancer-Related Mutant Forms of p53. *Cold Spring Harb Perspect Med*, *7*(2). https://doi.org/10.1101/cshperspect.a026054

Philips, F. S., Schwartz, H. S., Sternberg, S. S., & Tan, C. T. (1960). The toxicity of actinomycin D. *Ann N Y Acad Sci*, *89*, 348-360. https://doi.org/10.1111/j.1749-6632.1960.tb20158.x

Pietsch, E. C., Perchiniak, E., Canutescu, A. A., Wang, G., Dunbrack, R. L., & Murphy, M. E. (2008). Oligomerization of BAK by p53 utilizes conserved residues of the p53 DNA binding domain. *J Biol Chem*, *283*(30), 21294-21304. https://doi.org/10.1074/jbc.M710539200

Pignon, J. P., Arriagada, R., Ihde, D. C., Johnson, D. H., Perry, M. C., Souhami, R. L., Brodin, O., Joss, R. A., Kies, M. S., Lebeau, B., & et al. (1992). A meta-analysis of thoracic radiotherapy for small-cell lung cancer. *N Engl J Med*, *327*(23), 1618-1624. https://doi.org/10.1056/nejm199212033272302

Pijls-Johannesma, M., De Ruysscher, D., Vansteenkiste, J., Kester, A., Rutten, I., & Lambin, P. (2007). Timing of chest radiotherapy in patients with limited stage small cell lung cancer: A systematic review and meta-analysis of randomised controlled trials. *Cancer Treat Rev*, *33*(5), 461-473. https://doi.org/10.1016/j.ctrv.2007.03.002

Planchard, D., Groen, H., Kim, T., Rigas, J., Souquet, P., Baik, C., Barlesi, F., Mazières, J., Quoix, E., Curtis, C., Mookerjee, B., Bartlett-Pandite, A., Tucker, C., D'Amelio, A., & Johnson, B. (2015). Interim results of a phase II study of the BRAF inhibitor (BRAFi) dabrafenib (D) in combination with the MEK inhibitor trametinib (T) in patients (pts)

with BRAF V600E mutated (mut) metastatic non-small cell lung cancer (NSCLC). *J Clin Oncol 33: Abstract, 33*. https://doi.org/10.1200/jco.2015.33.15_suppl.8006

Podhorecka, M., Skladanowski, A., & Bozko, P. (2010). H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids, 2010*. https://doi.org/10.4061/2010/920161

Poeta, M. L., Manola, J., Goldwasser, M. A., Forastiere, A., Benoit, N., Califano, J. A., Ridge, J. A., Goodwin, J., Kenady, D., Saunders, J., Westra, W., Sidransky, D., & Koch, W. M. (2007). TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *N Engl J Med*, *357*(25), 2552-2561. https://doi.org/10.1056/NEJMoa073770

Postow, M. A., Callahan, M. K., & Wolchok, J. D. (2015). Immune Checkpoint Blockade in Cancer Therapy. *J Clin Oncol*, *33*(17), 1974-1982. https://doi.org/10.1200/jco.2014.59.4358

Preoperative chemotherapy for non-small-cell lung cancer: A systematic review and meta-analysis of individual participant data. (2014). *Lancet*, *383*(9928), 1561-1571. https://doi.org/10.1016/s0140-6736(13)62159-5

Ramesh, R., Munshi, A., & Panda, S. K. (1992). Polymerase chain reaction. *Natl Med J India*, *5*(3), 115-119.

Ramos, H., Raimundo, L., & Saraiva, L. (2020). p73: From the p53 shadow to a major pharmacological target in anticancer therapy. *Pharmacol Res*, *162*, 105245. https://doi.org/10.1016/j.phrs.2020.105245

Rao, B., Lain, S., & Thompson, A. M. (2013). p53-Based cyclotherapy: Exploiting the 'guardian of the genome' to protect normal cells from cytotoxic therapy. *Br J Cancer*, *109*(12), 2954-2958. https://doi.org/10.1038/bjc.2013.702

Rao, B., van Leeuwen, I. M., Higgins, M., Campbel, J., Thompson, A. M., Lane, D. P., & Lain, S. (2010). Evaluation of an Actinomycin D/VX-680 aurora kinase inhibitor combination in p53-based cyclotherapy. *Oncotarget*, *1*(7), 639-650. https://doi.org/10.18632/oncotarget.101015

Raso, M. G., Bota-Rabassedas, N., & Wistuba, II. (2021). Pathology and Classification of SCLC. *Cancers (Basel)*, *13*(4). https://doi.org/10.3390/cancers13040820

Ray, R. M., Bhattacharya, S., & Johnson, L. R. (2011). Mdm2 inhibition induces apoptosis in p53 deficient human colon cancer cells by activating p73- and E2F1-mediated expression of PUMA and Siva-1. *Apoptosis*, *16*(1), 35-44. https://doi.org/10.1007/s10495-010-0538-0

Ready, N. E., Ott, P. A., Hellmann, M. D., Zugazagoitia, J., Hann, C. L., de Braud, F., Antonia, S. J., Ascierto, P. A., Moreno, V., Atmaca, A., Salvagni, S., Taylor, M., Amin, A., Camidge, D. R., Horn, L., Calvo, E., Li, A., Lin, W. H., Callahan, M. K., & Spigel, D. R. (2020). Nivolumab Monotherapy and Nivolumab Plus Ipilimumab in Recurrent Small Cell Lung Cancer: Results From the CheckMate 032 Randomized Cohort. *J Thorac Oncol*, *15*(3), 426-435. https://doi.org/10.1016/j.jtho.2019.10.004

Reich, E. (1963). Biochemistry of Actinomycins. *Cancer Research*, *23*(8 Part 1), 1428-1441. https://cancerres.aacrjournals.org/content/canres/23/8_Part_1/1428.full.pdf

Reich, E., & Goldberg, I. H. (1964). Actinomycin and nucleic acid function. *Prog Nucleic Acid Res Mol Biol*, *3*, 183-234. https://doi.org/10.1016/s0079-6603(08)60742-4

Reich, E., Goldberg, I. H., & Rabinowitz, M. (1962). Structure-Activity Correlations of Actinomycins and their Derivatives. *Nature*, *196*(4856), 743-748. https://doi.org/10.1038/196743a0

Rieger, A. M., Nelson, K. L., Konowalchuk, J. D., & Barreda, D. R. (2011). Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. *J Vis Exp*(50). https://doi.org/10.3791/2597

Roskoski, R., Jr. (2019). Small molecule inhibitors targeting the EGFR/ErbB family of protein-tyrosine kinases in human cancers. *Pharmacol Res*, *139*, 395-411. https://doi.org/10.1016/j.phrs.2018.11.014

Ross, J. S., Wang, K., Elkadi, O. R., Tarasen, A., Foulke, L., Sheehan, C. E., Otto, G. A., Palmer, G., Yelensky, R., Lipson, D., Chmielecki, J., Ali, S. M., Elvin, J., Morosini, D., Miller, V. A., & Stephens, P. J. (2014). Next-generation sequencing reveals frequent consistent genomic alterations in small cell undifferentiated lung cancer. *J Clin Pathol*, *67*(9), 772-776. https://doi.org/10.1136/jclinpath-2014-202447

Roth, J., Dittmer, D., Rea, D., Tartaglia, J., Paoletti, E., & Levine, A. J. (1996). p53 as a target for cancer vaccines: recombinant canarypox virus vectors expressing p53 protect mice against lethal tumor cell challenge. *Proc Natl Acad Sci U S A*, *93*(10), 4781-4786. https://doi.org/10.1073/pnas.93.10.4781

Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., & Boyd, M. R. (1990). Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst*, *82*(13), 1113-1118. https://doi.org/10.1093/jnci/82.13.1113

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site

analysis for diagnosis of sickle cell anemia. *Science*, *230*(4732), 1350-1354. https://doi.org/10.1126/science.2999980

Samet, J. M., Avila-Tang, E., Boffetta, P., Hannan, L. M., Olivo-Marston, S., Thun, M. J., & Rudin, C. M. (2009). Lung cancer in never smokers: Clinical epidemiology and environmental risk factors. *Clin Cancer Res*, *15*(18), 5626-5645. https://doi.org/10.1158/1078-0432.Ccr-09-0376

Scharovsky, O. G., Mainetti, L. E., & Rozados, V. R. (2009). Metronomic chemotherapy: Changing the paradigm that more is better. *Curr Oncol*, *16*(2), 7-15. https://doi.org/10.3747/co.v16i2.420

Schiller, J. S., Lucas, J. W., Ward, B. W., & Peregoy, J. A. (2012). Summary health statistics for U.S. adults: National Health Interview Survey, 2010. *Vital Health Stat 10*(252), 1-207.

Schuler, M., & Green, D. R. (2001). Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans*, *29*(Pt 6), 684-688. https://doi.org/10.1042/0300-5127:0290684

Schwartz, A. G., & Cote, M. L. (2016). Epidemiology of Lung Cancer. *Adv Exp Med Biol*, *893*, 21-41. https://doi.org/10.1007/978-3-319-24223-1_2

Seca, A. M. L., & Pinto, D. (2018). Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. *Int J Mol Sci*, *19*(1). https://doi.org/10.3390/ijms19010263

Seiwert, T. Y., Zuo, Z., Keck, M. K., Khattri, A., Pedamallu, C. S., Stricker, T., Brown, C., Pugh, T. J., Stojanov, P., Cho, J., Lawrence, M. S., Getz, G., Brägelmann, J., DeBoer, R., Weichselbaum, R. R., Langerman, A., Portugal, L., Blair, E., Stenson, K., ... Hammerman, P. S. (2015). Integrative and Comparative Genomic Analysis of HPV-

Positive and HPV-Negative Head and Neck Squamous Cell Carcinomas. *Clinical Cancer Research*, *21*(3), 632-641. https://doi.org/10.1158/1078-0432.Ccr-13-3310

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, *88*(5), 593-602. https://doi.org/10.1016/s0092-8674(00)81902-9

Seute, T., Leffers, P., ten Velde, G. P., & Twijnstra, A. (2008). Detection of brain metastases from small cell lung cancer: consequences of changing imaging techniques (CT versus MRI). *Cancer*, *112*(8), 1827-1834. https://doi.org/10.1002/cncr.23361

Sharabi, A. B., Lim, M., DeWeese, T. L., & Drake, C. G. (2015). Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy. *Lancet Oncol*, *16*(13), e498-509. https://doi.org/10.1016/s1470-2045(15)00007-8

Shaw, A. T., Solomon, B. J., Chiari, R., Riely, G. J., Besse, B., Soo, R. A., Kao, S., Lin, C. C., Bauer, T. M., Clancy, J. S., Thurm, H., Martini, J. F., Peltz, G., Abbattista, A., Li, S., & Ou, S. I. (2019). Lorlatinib in advanced ROS1-positive non-small-cell lung cancer: A multicentre, open-label, single-arm, phase 1-2 trial. *Lancet Oncol*, *20*(12), 1691-1701. https://doi.org/10.1016/s1470-2045(19)30655-2

Shaw, A. T., Yeap, B. Y., Mino-Kenudson, M., Digumarthy, S. R., Costa, D. B., Heist, R. S., Solomon, B., Stubbs, H., Admane, S., McDermott, U., Settleman, J., Kobayashi, S., Mark, E. J., Rodig, S. J., Chirieac, L. R., Kwak, E. L., Lynch, T. J., & Iafrate, A. J. (2009). Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol*, *27*(26), 4247-4253. https://doi.org/10.1200/jco.2009.22.6993

Sheikh, M. S., Burns, T. F., Huang, Y., Wu, G. S., Amundson, S., Brooks, K. S., Fornace, A. J., Jr., & el-Deiry, W. S. (1998). p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res*, *58*(8), 1593-1598.

Shirole, N. H., Pal, D., Kastenhuber, E. R., Senturk, S., Boroda, J., Pisterzi, P., Miller, M., Munoz, G., Anderluh, M., Ladanyi, M., Lowe, S. W., & Sordella, R. (2017). TP53 exon-6 truncating mutations produce separation of function isoforms with pro-tumorigenic functions. *Elife*, *6*. https://doi.org/10.7554/eLife.25532

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer Statistics, 2021. *CA Cancer J Clin*, *71*(1), 7-33. https://doi.org/10.3322/caac.21654

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA Cancer J Clin*, *66*(1), 7-30. https://doi.org/10.3322/caac.21332

Singhal, G., & Rajeswari, M. R. (2009). Interaction of actinomycin D with promoter element of c-met and its inhibitory effect on the expression of c-Met. *J Biomol Struct Dyn*, *26*(5), 625-636. https://doi.org/10.1080/07391102.2009.10507277

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., & Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst*, *82*(13), 1107-1112. https://doi.org/10.1093/jnci/82.13.1107

Sneddon, J. B., Zhen, H. H., Montgomery, K., van de Rijn, M., Tward, A. D., West, R., Gladstone, H., Chang, H. Y., Morganroth, G. S., Oro, A. E., & Brown, P. O. (2006). Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-

associated stromal cells and can promote tumor cell proliferation. *Proc Natl Acad Sci U S A*, *103*(40), 14842-14847. https://doi.org/10.1073/pnas.0606857103

Soda, M., Choi, Y. L., Enomoto, M., Takada, S., Yamashita, Y., Ishikawa, S., Fujiwara, S., Watanabe, H., Kurashina, K., Hatanaka, H., Bando, M., Ohno, S., Ishikawa, Y., Aburatani, H., Niki, T., Sohara, Y., Sugiyama, Y., & Mano, H. (2007). Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*, *448*(7153), 561-566. https://doi.org/10.1038/nature05945

Solassol, I., Pinguet, F., & Quantin, X. (2019). FDA- and EMA-Approved Tyrosine Kinase Inhibitors in Advanced EGFR-Mutated Non-Small Cell Lung Cancer: Safety, Tolerability, Plasma Concentration Monitoring, and Management. *Biomolecules*, *9*(11). https://doi.org/10.3390/biom9110668

Solomon, B., Young, R. J., & Rischin, D. (2018). Head and neck squamous cell carcinoma: Genomics and emerging biomarkers for immunomodulatory cancer treatments. *Semin Cancer Biol*, *52*(Pt 2), 228-240. https://doi.org/10.1016/j.semcancer.2018.01.008

Speidel, D. (2010). Transcription-independent p53 apoptosis: An alternative route to death. *Trends Cell Biol*, *20*(1), 14-24. https://doi.org/10.1016/j.tcb.2009.10.002

Speidel, D., Helmbold, H., & Deppert, W. (2006). Dissection of transcriptional and non-transcriptional p53 activities in the response to genotoxic stress. *Oncogene*, *25*(6), 940-953. https://doi.org/10.1038/sj.onc.1209126

Spreafico, F., & Bellani, F. F. (2006). Wilms' tumor: past, present and (possibly) future. *Expert Rev Anticancer Ther*, *6*(2), 249-258. https://doi.org/10.1586/14737140.6.2.249

Stiewe, T., & Pützer, B. M. (2000). Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nat Genet*, *26*(4), 464-469. https://doi.org/10.1038/82617

Szturz, P., & Vermorken, J. B. (2016). Treatment of Elderly Patients with Squamous Cell Carcinoma of the Head and Neck. *Front Oncol*, *6*, 199. https://doi.org/10.3389/fonc.2016.00199

Taira, N., Nihira, K., Yamaguchi, T., Miki, Y., & Yoshida, K. (2007). DYRK2 Is Targeted to the Nucleus and Controls p53 via Ser46 Phosphorylation in the Apoptotic Response to DNA Damage. *Molecular Cell*, *25*(5), 725-738. https://doi.org/https://doi.org/10.1016/j.molcel.2007.02.007

Taylor, J. T., Ellison, S., Pandele, A., Wood, S., Nathan, E., Forte, G., Parker, H., Zindy, E., Elvin, M., Dickson, A., Williams, K. J., Karabatsou, K., McCabe, M., McBain, C., & Bigger, B. W. (2020). Actinomycin D downregulates Sox2 and improves survival in preclinical models of recurrent glioblastoma. *Neuro Oncol*, *22*(9), 1289-1301. https://doi.org/10.1093/neuonc/noaa051

Timmerman, R., Paulus, R., Galvin, J., Michalski, J., Straube, W., Bradley, J., Fakiris, A., Bezjak, A., Videtic, G., Johnstone, D., Fowler, J., Gore, E., & Choy, H. (2010). Stereotactic body radiation therapy for inoperable early stage lung cancer. *Jama*, *303*(11), 1070-1076. https://doi.org/10.1001/jama.2010.261

Timofeev, O., Klimovich, B., Schneikert, J., Wanzel, M., Pavlakis, E., Noll, J., Mutlu, S., Elmshäuser, S., Nist, A., Mernberger, M., Lamp, B., Wenig, U., Brobeil, A., Gattenlöhner, S., Köhler, K., & Stiewe, T. (2019). Residual apoptotic activity of a tumorigenic p53 mutant improves cancer therapy responses. *Embo j*, *38*(20), e102096. https://doi.org/10.15252/embj.2019102096

Torre, L. A., Siegel, R. L., & Jemal, A. (2016). Lung Cancer Statistics. *Adv Exp Med Biol*, *893*, 1-19. https://doi.org/10.1007/978-3-319-24223-1_1

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A, 76*(9), 4350-4354. https://doi.org/10.1073/pnas.76.9.4350

Travis, W. D. (2012). Update on small cell carcinoma and its differentiation from squamous cell carcinoma and other non-small cell carcinomas. *Mod Pathol, 25 Suppl 1*, S18-30. https://doi.org/10.1038/modpathol.2011.150

Travis, W. D., Asamura, H., Bankier, A. A., Beasley, M. B., Detterbeck, F., Flieder, D. B., Goo, J. M., MacMahon, H., Naidich, D., Nicholson, A. G., Powell, C. A., Prokop, M., Rami-Porta, R., Rusch, V., van Schil, P., & Yatabe, Y. (2016). The IASLC Lung Cancer Staging Project: Proposals for Coding T Categories for Subsolid Nodules and Assessment of Tumor Size in Part-Solid Tumors in the Forthcoming Eighth Edition of the TNM Classification of Lung Cancer. *J Thorac Oncol, 11*(8), 1204-1223. https://doi.org/10.1016/j.jtho.2016.03.025

Travis, W. D., Travis, L. B., & Devesa, S. S. (1995). Lung cancer. *Cancer, 75*(1 Suppl), 191-202. https://doi.org/10.1002/1097-0142(19950101)75:1+<191::aid-cncr2820751307>3.0.co;2-y

Turrisi, A. T., 3rd, Kim, K., Blum, R., Sause, W. T., Livingston, R. B., Komaki, R., Wagner, H., Aisner, S., & Johnson, D. H. (1999). Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. *N Engl J Med, 340*(4), 265-271. https://doi.org/10.1056/nejm199901283400403

Tzaridis, T., Milde, T., Pajtler, K. W., Bender, S., Jones, D. T., Müller, S., Wittmann, A., Schlotter, M., Kulozik, A. E., Lichter, P., Peter Collins, V., Witt, O., Kool, M.,

Korshunov, A., Pfister, S. M., & Witt, H. (2016). Low-dose Actinomycin-D treatment re-establishes the tumoursuppressive function of P53 in RELA-positive ependymoma. *Oncotarget*, *7*(38), 61860-61873. https://doi.org/10.18632/oncotarget.11452

Vaddepally, R. K., Kharel, P., Pandey, R., Garje, R., & Chandra, A. B. (2020). Review of Indications of FDA-Approved Immune Checkpoint Inhibitors per NCCN Guidelines with the Level of Evidence. *Cancers (Basel)*, *12*(3). https://doi.org/10.3390/cancers12030738

Valente, L. J., Gray, D. H., Michalak, E. M., Pinon-Hofbauer, J., Egle, A., Scott, C. L., Janic, A., & Strasser, A. (2013). p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa. *Cell Rep*, *3*(5), 1339-1345. https://doi.org/10.1016/j.celrep.2013.04.012

van den Heuvel-Eibrink, M. M., Hol, J. A., Pritchard-Jones, K., van Tinteren, H., Furtwängler, R., Verschuur, A. C., Vujanic, G. M., Leuschner, I., Brok, J., Rübe, C., Smets, A. M., Janssens, G. O., Godzinski, J., Ramírez-Villar, G. L., de Camargo, B., Segers, H., Collini, P., Gessler, M., Bergeron, C., ... Graf, N. (2017). Position paper: Rationale for the treatment of Wilms tumour in the UMBRELLA SIOP-RTSG 2016 protocol. *Nat Rev Urol*, *14*(12), 743-752. https://doi.org/10.1038/nrurol.2017.163

van Leeuwen, I. M., Rao, B., Sachweh, M. C., & Laín, S. (2012). An evaluation of small-molecule p53 activators as chemoprotectants ameliorating adverse effects of anticancer drugs in normal cells. *Cell Cycle*, *11*(9), 1851-1861. https://doi.org/10.4161/cc.20254

Vansteenkiste, J., Crinò, L., Dooms, C., Douillard, J. Y., Faivre-Finn, C., Lim, E., Rocco, G., Senan, S., Van Schil, P., Veronesi, G., Stahel, R., Peters, S., & Felip, E. (2014). 2nd ESMO Consensus Conference on Lung Cancer: Early-stage non-small-cell lung cancer

consensus on diagnosis, treatment and follow-up. *Ann Oncol*, *25*(8), 1462-1474. https://doi.org/10.1093/annonc/mdu089

Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., & Liu, E. A. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, *303*(5659), 844-848. https://doi.org/10.1126/science.1092472

Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*, *184*(1), 39-51. https://doi.org/10.1016/0022-1759(95)00072-i

Vermeulen, L., De Sousa, E. M. F., van der Heijden, M., Cameron, K., de Jong, J. H., Borovski, T., Tuynman, J. B., Todaro, M., Merz, C., Rodermond, H., Sprick, M. R., Kemper, K., Richel, D. J., Stassi, G., & Medema, J. P. (2010). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol*, *12*(5), 468-476. https://doi.org/10.1038/ncb2048

Vermorken, J. B., Mesia, R., Rivera, F., Remenar, E., Kawecki, A., Rottey, S., Erfan, J., Zabolotnyy, D., Kienzer, H. R., Cupissol, D., Peyrade, F., Benasso, M., Vynnychenko, I., De Raucourt, D., Bokemeyer, C., Schueler, A., Amellal, N., & Hitt, R. (2008). Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med*, *359*(11), 1116-1127. https://doi.org/10.1056/NEJMoa0802656

Vermorken, J. B., Trigo, J., Hitt, R., Koralewski, P., Diaz-Rubio, E., Rolland, F., Knecht, R., Amellal, N., Schueler, A., & Baselga, J. (2007). Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in

patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. *J Clin Oncol*, *25*(16), 2171-2177. https://doi.org/10.1200/jco.2006.06.7447

Villalobos, P., & Wistuba, II. (2017). Lung Cancer Biomarkers. *Hematol Oncol Clin North Am*, *31*(1), 13-29. https://doi.org/10.1016/j.hoc.2016.08.006

Voigt, W. (2005). Sulforhodamine B assay and chemosensitivity. *Methods Mol Med*, *110*, 39-48. https://doi.org/10.1385/1-59259-869-2:039

von Pawel, J., Jotte, R., Spigel, D. R., O'Brien, M. E., Socinski, M. A., Mezger, J., Steins, M., Bosquée, L., Bubis, J., Nackaerts, K., Trigo, J. M., Clingan, P., Schütte, W., Lorigan, P., Reck, M., Domine, M., Shepherd, F. A., Li, S., & Renschler, M. F. (2014). Randomized phase III trial of amrubicin versus topotecan as second-line treatment for patients with small-cell lung cancer. *J Clin Oncol*, *32*(35), 4012-4019. https://doi.org/10.1200/jco.2013.54.5392

Vousden, K. H., & Lane, D. P. (2007). p53 in health and disease. *Nat Rev Mol Cell Biol*, *8*(4), 275-283. https://doi.org/10.1038/nrm2147

Vredenburgh, J. J., Desjardins, A., Herndon, J. E., 2nd, Dowell, J. M., Reardon, D. A., Quinn, J. A., Rich, J. N., Sathornsumetee, S., Gururangan, S., Wagner, M., Bigner, D. D., Friedman, A. H., & Friedman, H. S. (2007). Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. *Clin Cancer Res*, *13*(4), 1253-1259. https://doi.org/10.1158/1078-0432.Ccr-06-2309

Waksman, S., & Woodruff, H. B. (1940). Bacteriostatic and Bactericidal Substances Produced by a Soil Actinomyces. . *Proceedings of the Society for Experimental Biology and Medicine*, *45*, 609 - 614.

Waksman, S. A., & Woodruff, H. B. (1940). Bacteriostatic and Bactericidal Substances Produced by a Soil Actinomyces. *Proceedings of the Society for Experimental Biology and Medicine*, *45*(2), 609-614. https://doi.org/10.3181/00379727-45-11768

Wang, R., Hu, H., Pan, Y., Li, Y., Ye, T., Li, C., Luo, X., Wang, L., Li, H., Zhang, Y., Li, F., Lu, Y., Lu, Q., Xu, J., Garfield, D., Shen, L., Ji, H., Pao, W., Sun, Y., & Chen, H. (2012). RET fusions define a unique molecular and clinicopathologic subtype of non-small-cell lung cancer. *J Clin Oncol*, *30*(35), 4352-4359. https://doi.org/10.1200/jco.2012.44.1477

Wang, S., & El-Deiry, W. S. (2003). TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene*, *22*(53), 8628-8633. https://doi.org/10.1038/sj.onc.1207232

Wang, S., Zimmermann, S., Parikh, K., Mansfield, A. S., & Adjei, A. A. (2019). Current Diagnosis and Management of Small-Cell Lung Cancer. *Mayo Clin Proc*, *94*(8), 1599-1622. https://doi.org/10.1016/j.mayocp.2019.01.034

Warde, P., & Payne, D. (1992). Does thoracic irradiation improve survival and local control in limited-stage small-cell carcinoma of the lung? A meta-analysis. *J Clin Oncol*, *10*(6), 890-895. https://doi.org/10.1200/jco.1992.10.6.890

Weissmueller, S., Manchado, E., Saborowski, M., Morris, J. P. t., Wagenblast, E., Davis, C. A., Moon, S. H., Pfister, N. T., Tschaharganeh, D. F., Kitzing, T., Aust, D., Markert, E. K., Wu, J., Grimmond, S. M., Pilarsky, C., Prives, C., Biankin, A. V., & Lowe, S. W. (2014). Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor β signaling. *Cell*, *157*(2), 382-394. https://doi.org/10.1016/j.cell.2014.01.066

Winquist, E., Agbassi, C., Meyers, B. M., Yoo, J., & Chan, K. K. W. (2017). Systemic therapy in the curative treatment of head and neck squamous cell cancer: A systematic review. *J Otolaryngol Head Neck Surg*, *46*(1), 29. https://doi.org/10.1186/s40463-017-0199-x

Wolchok, J. D., Kluger, H., Callahan, M. K., Postow, M. A., Rizvi, N. A., Lesokhin, A. M., Segal, N. H., Ariyan, C. E., Gordon, R. A., Reed, K., Burke, M. M., Caldwell, A., Kronenberg, S. A., Agunwamba, B. U., Zhang, X., Lowy, I., Inzunza, H. D., Feely, W., Horak, C. E., ...Sznol, M. (2013). Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*, *369*(2), 122-133. https://doi.org/10.1056/NEJMoa1302369

Woodard, G. A., Jones, K. D., & Jablons, D. M. (2016). Lung Cancer Staging and Prognosis. *Cancer Treat Res*, *170*, 47-75. https://doi.org/10.1007/978-3-319-40389-2_3

Xu, H., & Krystal, G. W. (2010). Actinomycin D decreases Mcl-1 expression and acts synergistically with ABT-737 against small cell lung cancer cell lines. *Clin Cancer Res*, *16*(17), 4392-4400. https://doi.org/10.1158/1078-0432.Ccr-10-0640

Yin, H., Jiang, Z., Wang, S., & Zhang, P. (2019). Actinomycin D-Activated RNase L Promotes H2A.X/H2B-Mediated DNA Damage and Apoptosis in Lung Cancer Cells [Original Research]. *Frontiers in Oncology*, *9*(1086). https://doi.org/10.3389/fonc.2019.01086

Yu, J., & Zhang, L. (2005). The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun*, *331*(3), 851-858. https://doi.org/10.1016/j.bbrc.2005.03.189

Zembruski, N. C. L., Stache, V., Haefeli, W. E., & Weiss, J. (2012). 7-Aminoactinomycin D for apoptosis staining in flow cytometry. *Analytical Biochemistry*, *429*(1), 79-81. https://doi.org/https://doi.org/10.1016/j.ab.2012.07.005

Zhang, Q., Shi, S., Yen, Y., Brown, J., Ta, J. Q., & Le, A. D. (2010). A subpopulation of CD133+ cancer stem-like cells characterized in human oral squamous cell carcinoma confer resistance to chemotherapy. *Cancer Letters*, *289*(2), 151-160. https://doi.org/https://doi.org/10.1016/j.canlet.2009.08.010

Zhang, X., Tang, N., Hadden, T. J., & Rishi, A. K. (2011). Akt, FoxO and regulation of apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1813*(11), 1978-1986. https://doi.org/https://doi.org/10.1016/j.bbamcr.2011.03.010

Zhao, M., Sano, D., Pickering, C. R., Jasser, S. A., Henderson, Y. C., Clayman, G. L., Sturgis, E. M., Ow, T. J., Lotan, R., Carey, T. E., Sacks, P. G., Grandis, J. R., Sidransky, D., Heldin, N. E., & Myers, J. N. (2011). Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res*, *17*(23), 7248-7264. https://doi.org/10.1158/1078-0432.Ccr-11-0690

APPENDIX A: IRB LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

May 10, 2021

Adeoluwa Adeloula
2101 Buffington Ave, Apt 136
Huntington, WV 25703

Dear Adeoluwa:

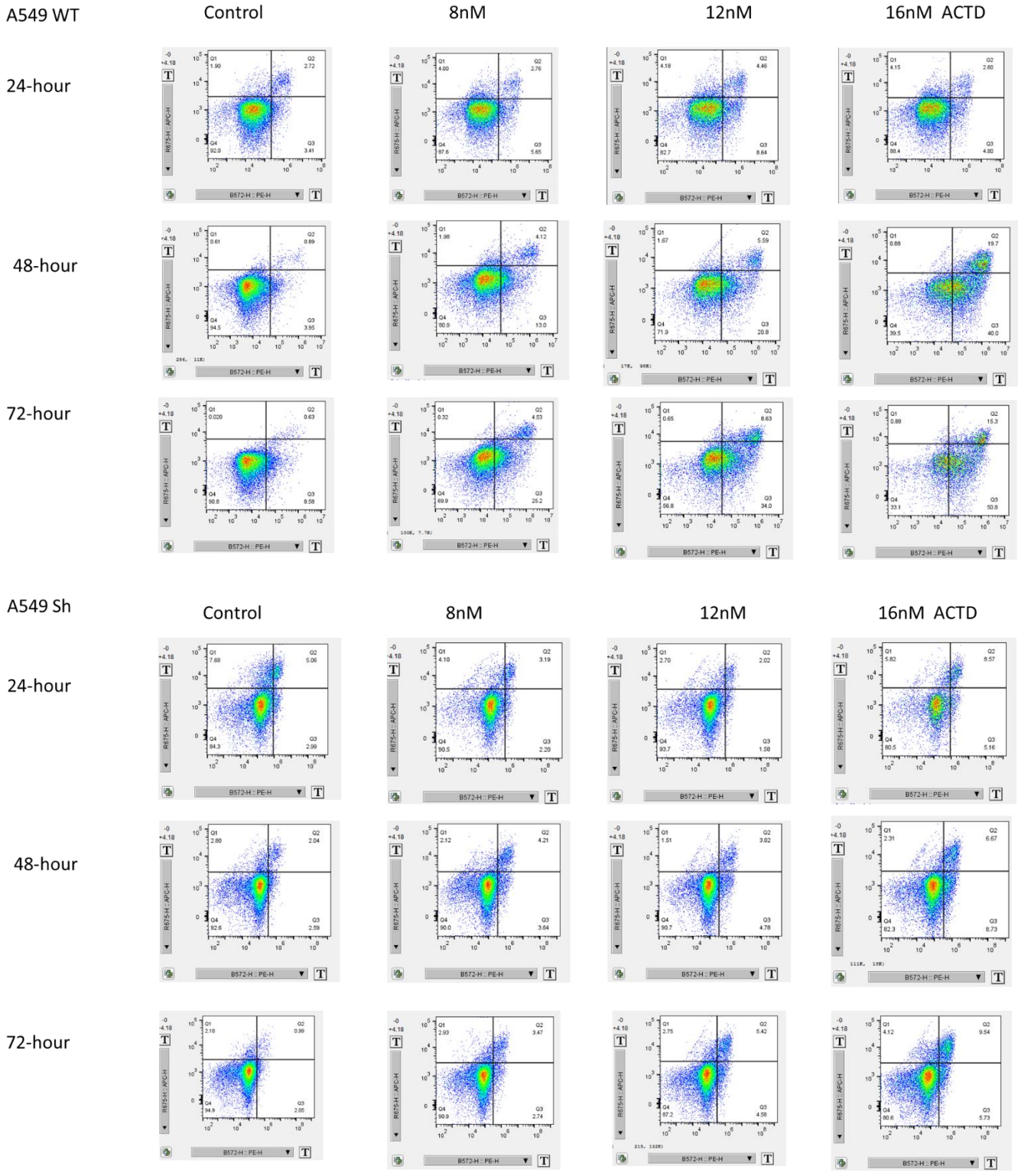
This letter is in response to the submitted thesis abstract entitled *"Mechanisms of Apoptosis-induced by Actinomycin D in Aerodigestive Tract Cancers."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

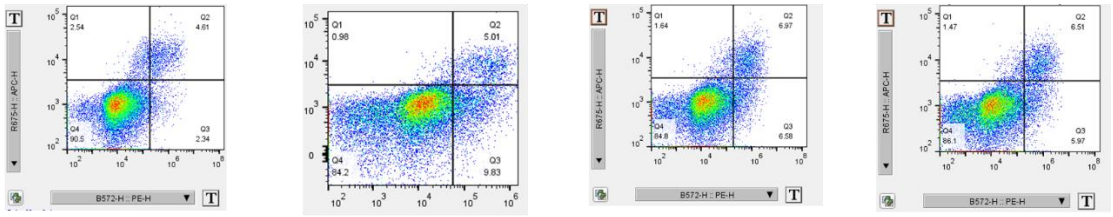
APPENDIX B: REPRESENTATIVE APOPTOTIC PLOTS



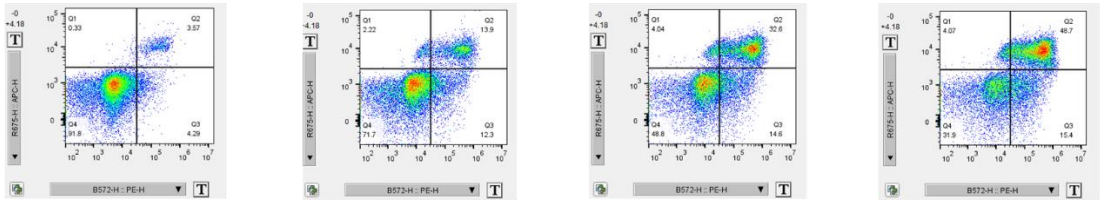
H460WT

Control 4nM 6nM 8nM ACTD

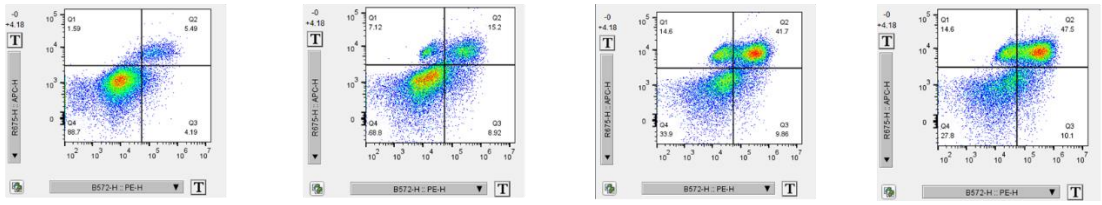
24-hour



48-hour



72-hour



H460Sh

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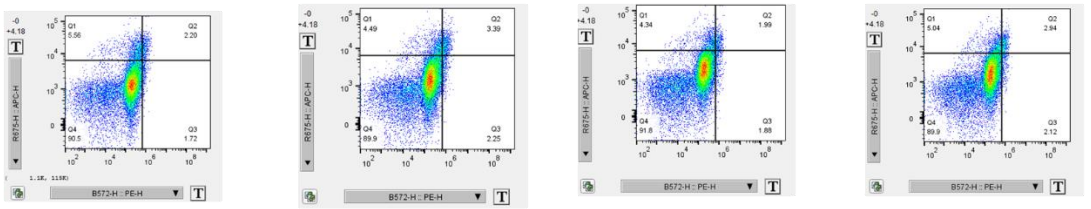
24-hour



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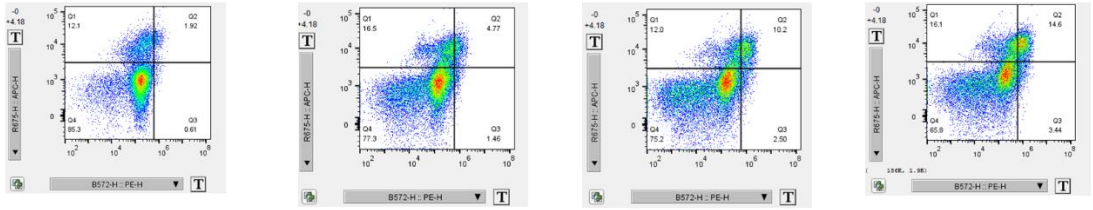

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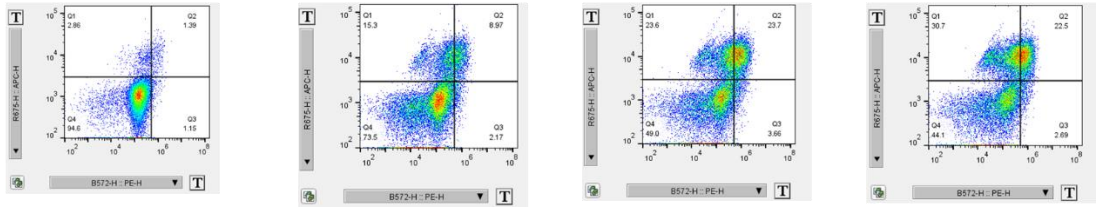
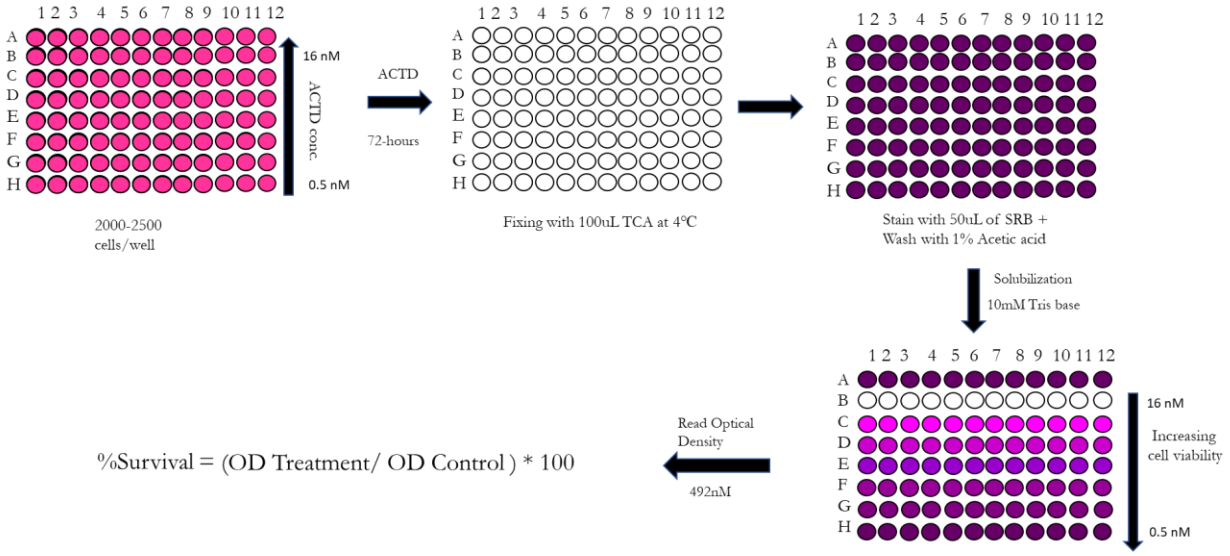


APPENDIX C: SRB ASSAY FLOWCHART



APPENDIX D: ABBREVIATIONS

7-AAD	- 7-Aminoactinomycin D
ACTD	- Actinomycin D
ALDH1	- Aldehyde dehydrogenase 1
ALK	- Anaplastic lymphoma kinase
APAF-1	- Apoptotic protease activating factor
ATM	- Ataxia telangiectasia mutated
BCL6	- B-cell lymphoma 6
BCR	- Breakpoint cluster
BRCA2	- Breast Cancer Type 2 susceptibility protein
BSA	- Bovine serum albumin
CCDN1	- Cyclin D1
CD133	- Prominin-1
CDKN2	- Cyclin dependent kinase inhibitor 2
CRT	- Chemoradiotherapy
CSC	- Cancer Stem Cell
CTLA4	- Cytotoxic T-lymphocyte associated protein 4
DISC	- Death-inducing signaling complex
DMEM	- Dulbecco’s modified eagle medium
DMSO	- Dimethylsulfoxide
DNA	- Deoxyribonucleic acid
DR4	- Death receptor 4
DR5	- Death receptor 5

EDTA - Ethylenediaminetetraacetic acid

EGFR - Epidermal growth factor receptor

EMA - European medicines agency

EML4 - Echinoderm microtubule-associated protein-like 4

FADD - FAS-associated via death domain

FBS - Fetal bovine serum

FDA - Food and drugs administration

FGFR - Fibroblast growth factor receptor

FGFR1 - Fibroblast growth factor receptor 1

FOXO - Forkhead box O

HER2 - Human epidermal growth factor receptor

HPV - Human papilloma virus

ICR - Immune checkpoint receptor -

MAPK - Mitogen activated protein kinase

MCL1 - Induced myeloid leukemia cell differentiation protein

MDM2 - Mouse double minute 2

MOMP - Mitochondrial outer membrane permeabilisation

mTOR - Mammalian target of rapamycin

NCI - National Cancer Institute

NF1 - Neurofibromatosis type 1

NSCLC - Non-small cell lung cancer

OCT3 - Octamer-binding transcription factor 3

OCT4 - Octamer-binding transcription factor 4

PAF	- PCNA-associated factor
PAI1	- Plasminogen activator inhibitor 1
PARP	- Poly (ADP-ribose) polymerase
PBS	- Phosphate buffered saline
PCI	- Prophylactic cranial irradiation
PD1	- Programmed cell death protein 1
PD-L1	- Programmed death-ligand 1
PI	- Propidium iodide
PI3K	- Phosphoinositide 3-kinase/ Phosphatidylinositol 3-kinase
PI3KCA	- Phophatidylinositol 3-kinase catalytic subunit alpha isoform
PS	- Phosphatidylserine
PTEN	- Phosphatase and tensin homolog
PTMs	- Post-translational modifications
PUMA	- p53 upregulated modulator apoptosis
PVDF	- Polyvinylidene fluoride
qPCR	- Quantitative polymerase chain reaction
RAF	- Rapidly accelerated fibrosarcoma
RB1	- RB Transcriptional Corepressor 1
RICTOR	- Rapamycin-insensitive companion of mammalian target of rapamycin
RNA	- Ribonucleic acid
ROS1	- Reactive oxygen species
RPMI	- Roswell park memorial institute medium
SCC	- Squamous cell carcinoma

SCCHN	- Squamous cell carcinoma of the head and neck
SCLC	- Small cell lung cancer
SDS	- Sodium dodecyl sulfate
SDS-PAGE	- Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SMAC/DIABLO	- Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
SOX2	- (Sex determining region Y)-box 2
SRB	- Sulforhodamine B
TCGA	- The cancer genomic atlas
TIL	- Tumor infiltrating lymphocytes
TKI	- Tyrosine kinase inhibitor
TRAF3	- TNF receptor associated factor
TRAIL	- TNF-related apoptosis-inducing ligand (TRAIL)
TSP1	- Thrombospondin-1
VA	- Veterans Affairs
VEGF	- Vascular endothelial factor