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E6 Inhibtion-Mediated Combinatorial Therapy for HPV+ HNSCC

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E6 Inhibition-Mediated Combinatorial Therapy for HPV^{+} HNSCC

By

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APPROVAL PAGE

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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DEDICATION

I'd like to dedicate this thesis to my father and my grandmother who have passed away from cancer and have inspired me to pursue my studies in cancer research, and have in addition, been a spiritual light in my life.

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LIST OF ABBREVIATIONS

ACF	Animal care facility
ALT	Alanine transaminase
APC	Antigen-presenting cells
AST	Aspartate aminotransferase
BCG	Bacille Calmette-Guerin
Bcl-2	B-cell lymphoma 2
BLI	Bioluminescence intensity
CDDP	cis-Diammineplatinum (II) dichloride, Cisplatin
CK	Creatine kinase
CO_2	Carbon dioxide
CYP	Cytochrome P450
DC	Dendritic cells
DED	Death effector domains
DISC	Death-inducing signaling complex
DR	Death receptor
ECOG	Eastern Cooperative Oncology Group
eGFP	Enhanced green fluorescence protein
EGFR	Epidermal growth factor receptor
FADD	Fas-associated death domain
FDA	Food and Drug Administration
ffLuc	*Firefly luciferase*

GA	Gambogic acid
GA-OH	30-hydroxygambogic acid
GLSR	Generalized least squares regression
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR-HPV	High-risk human papillomavirus
hrTRAIL	Human recombinant TRAIL
Hsp	Heat shock protein
HTS	High-throughput screening
IACUC	Institutional Animal Care and Use Committee
IARC	International Agency for Research against Cancer
IDLV	Integrase defective lentiviral vector
Ig	Immunoglobulin
IHC	Immunohistochemistry
IMRT	Intensity modulated radiotherapy
ISH	*in situ* hybridization
ITC	Isothermal scanning calorimetry
L	Length
mAb	Monoclonal antibodies
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin

NK	Natural killer
OPSCC	Oropharynx squamous cell carcinoma
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
pRb	Retinoblastoma protein
RCT	Radiochemotherapy
RITA	Reactivation of p53 and Induction of Tumor cell Apoptosis
ROI	Region of interest
RTOG	Radiation Therapy Oncology Group
SCC	Squamous cell carcinoma
SE	Standard error
siRNA	Small interfering RNA
TKIs	Tyrosine kinase inhibitors
TLR	Toll-like receptor
TMS	Transoral laser microsurgery
TNF	Tumor necrosis factor
TORS	Transoral robotic surgery
TOS	Trans-oral surgery
TRAIL	TNF-related apoptosis-inducing ligand
V	Volume
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

ABSTRACT OF THE DISSERTATION

E6 Inhibition-Mediated Combinatorial Therapy for HPV$^+$ HNSCC

by

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Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, June 2024
Dr. Penelope Duerksen-Hughes, Principal Investigator

High-risk human papillomaviruses (HPV) are the causative agents of virtually all cases of cervical cancer and up to 80% of head and neck squamous cell carcinoma (HNSCC). Current treatment options include chemo- and radiotherapy, though the effectiveness of these therapies is limited by the viral oncoprotein E6, which disrupts apoptotic pathways by binding to and accelerating the degradation of molecules such as p53 and caspase-8. Our hypothesis was that inhibiting E6 and rescuing the apoptotic mediators would increase the effectiveness of apoptotic agents. The aims of this study were to first, develop an HPV$^+$ HNSCC xenograft model to test small E6 inhibiting molecules; and second, to evaluate the antitumor efficacy of lead molecules in an established *in vivo* model. We fulfilled the first aim by optimizing an HNSCC xenograft model using UM-SCC47 cells, incorporating Matrigel, and applying luciferase technology. Our lab has identified two E6 inhibitors from two different sources: spinacine was identified following screening of the TimTec Actiprobe 2K library, and 30-hydroxygambogic acid (GA-OH) was identified following screening of the Kansas University 5K library with subsequent follow-up. Both compounds were shown to rescue the apoptotic signaling molecules and to re-sensitize

cancer cells to apoptosis *in vitro*. To further explore the therapeutic potential of these small molecules, we determined their toxicity and antitumor efficacy *in vivo*. Spinacine exhibited no toxicity to mice at doses up to and including 20 mg/kg but did not yield evidence of efficacy on tumor growth either alone or when combined with apoptotic agents. Subsequently, our lab demonstrated that GA-OH had higher specificity and effectiveness than our previous lead molecules *in vitro*. Therefore, we assessed GA-OH's toxicity and evaluated the effectiveness of GA-OH in combination with chemotherapy. Behavioral/physical assessments, body weight, organ necropsy, and blood tests, with the possible exception of creatine kinase, all pointed towards lack of significance toxicity, predicting tolerance. Moreover, we were able to demonstrate that our animal model was functional, and that cetuximab slowed and regressed tumor growth. We found that GA-OH alone did not slow tumor growth; however, when combined with cisplatin, GA-OH enhanced cisplatin's efficacy in HPV^{+} HNSCC *in vivo.*

CHAPTER ONE

INTRODUCTION

Recent Progress in Therapeutic Treatments and Screening Strategies for the Prevention and Treatment of HPV-Associated Head and Neck Cancer

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Chapter One is adapted from Whang SN, Filippova M, Duerksen-Hughes P. Recent Progress in Therapeutic Treatments and Screening Strategies for the Prevention and Treatment of HPV-Associated Head and Neck Cancer. *Viruses. 2015 Sep 17;7(9):5040-65. doi: 10.3390/v7092860.*
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Abstract: The rise in human papillomavirus (HPV)-associated head and neck squamous cell carcinoma (HNSCC) has elicited significant interest in the role of high-risk HPV in tumorigenesis. Because patients with HPV-positive HNSCC have better prognoses than do their HPV-negative counterparts, current therapeutic strategies for HPV^+ HNSCC are increasingly considered to be overly aggressive, highlighting a need for customized treatment guidelines for this cohort. Additional issues include the unmet need for a reliable screening strategy for HNSCC, as well as the ongoing assessment of the efficacy of prophylactic vaccines for the prevention of HPV infections in the head and neck regions. This review also outlines a number of emerging prospects for therapeutic vaccines, as well as for targeted, molecular-based therapies for HPV-associated head and neck cancers. Overall, the future for developing novel and effective therapeutic agents for HPV-associated head and neck tumors is promising; continued progress is critical in order to meet the challenges posed by the growing epidemic.

Keywords: head and neck squamous cell carcinoma; high risk HPV; HPV-related oropharyngeal squamous cell carcinoma; cancer treatments; target therapy; HNSCC; HR HPV; OPSCC

1. Introduction and Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, with an incidence of over half a million new cases annually (Bol & Gregoire, 2014; Elrefaey, Massaro, Chiocca, Chiesa, & Ansarin, 2014; Friedman, Stavas, & Cmelak, 2014; Machiels et al., 2014; van Kempen et al., 2014). The most common tumor sites of HNSCC include the oral cavity, nasal cavity, larynx, hypopharynx, and the oropharynx (Chai, Lambie, Verma, & Punyadeera, 2015; Elrefaey et al., 2014; Friedman et al., 2014; John, Wu, Lee, & Farah, 2013; Machiels et al., 2014; Vermeer et al., 2013). A few decades ago, a decline in HNSCC in relation to the carcinomas of the hypopharynx and larynx was indicated (Boscolo-Rizzo et al., 2013; D'Souza & Dempsey, 2011; Elrefaey et al., 2014; Osazuwa-Peters et al., 2015; van Kempen et al., 2014). This was attributed to the rise in public awareness (Monnier & Simon, 2015; Moore & Mehta, 2015; Sepiashvili et al., 2015; Vermeer et al., 2013) and the consequential decline in excessive tobacco and alcohol consumption, factors traditionally associated with this carcinoma (Adams, Wise-Draper, & Wells, 2014; Antonsson et al., 2015; Arbyn et al., 2012; Elrefaey et al., 2014; van Kempen et al., 2014). In contrast to the encouraging trend, certain types of HNSCC have risen over the past couple of decades due to an increase in the incidence of oropharynx squamous cell carcinoma (OPSCC) (D'Souza & Dempsey, 2011; Elrefaey et al., 2014; Osazuwa-Peters et al., 2015; van Kempen et al., 2014), which includes cancers that form in the tonsils and at the base of the tongue (Adams et al., 2014; Arbyn et al., 2012; Bosch et al., 2013; Boscolo-Rizzo et al., 2013; D'Souza & Dempsey, 2011; Dalianis, 2014a; Machiels et al., 2014). This became particularly evident in patients with no history of

tobacco smoking or alcohol abuse (Antonsson et al., 2015; Ramshankar & Krishnamurthy, 2013; van Kempen et al., 2014), arguing for the presence of an additional etiological agent (Adams et al., 2014; Friedman et al., 2014; van Kempen et al., 2014). The striking increase in these cancers has been attributed to the rising prevalence of human papillomavirus (HPV)-associated tumors (Adams et al., 2014; Antonsson et al., 2015; Chaturvedi et al., 2011; D'Souza & Dempsey, 2011; Gillison et al., 2000; Psyrri, Sasaki, Vassilakopoulou, Dimitriadis, & Rampias, 2012; Urban, Corry, & Rischin, 2014).

The link between HPV and oropharyngeal carcinoma was initially suggested four decades ago, when it was still considered a risk factor (Adams et al., 2014; Osazuwa-Peters et al., 2015). However, it was not until the past decade that the prevalence of HPV in the head and neck has elicited considerable attention (Garbuglia, 2014), and the International Agency for Research against Cancer (IARC) has now acknowledged HPV as an emergent etiological factor in the development of OPSCC (Adams et al., 2014; Dalianis, 2014a, 2014b; Machiels et al., 2014; Osazuwa-Peters et al., 2015; Ramshankar & Krishnamurthy, 2013). With up to 80% of OPSCC now related to HPV (Dalianis, 2014b), research reveals that the virus has undoubtedly altered the epidemiology and survival outcome landscape of head and neck carcinoma (Chaturvedi et al., 2011; Dreyer, Hauck, Oliveira-Silva, Barros, & Niedobitek, 2013; Friedman et al., 2014). In fact, the incidence of HPV-negative head and neck squamous cell carcinomas has statistically decreased by 50%, in step with the gradual reduction of tobacco and alcohol use since the 1980s (Arbyn et al., 2012; Bonilla-Velez, Mroz, Hammon, & Rocco, 2013; Chaturvedi et al., 2011; Garbuglia, 2014; Osazuwa-Peters et al., 2015; Wierzbicka et al., 2013). In contrast, HPV-

positive oropharyngeal carcinomas have escalated by a dramatic 225% in the US (Arbyn et al., 2012; Bonilla-Velez et al., 2013; Chaturvedi et al., 2011; Garbuglia, 2014; Moore & Mehta, 2015; Osazuwa-Peters et al., 2015; Wierzbicka et al., 2013), and they will represent a large fraction of the HNSCC population in the country within the next 20 years (Boscolo-Rizzo et al., 2013; Chaturvedi et al., 2011). In fact, at the current rate of increase, OPSCC is predicted to surpass the incidence of HPV-positive cervical cancer, the archetypal HPV malignancy, in the US by the year 2020 (Adams et al., 2014; Bonilla-Velez et al., 2013; Caicedo-Granados et al., 2014; Chaturvedi et al., 2011; Elrefaey et al., 2014; Lui & Grandis, 2012; Moore & Mehta, 2015; Psyrri et al., 2012; Rettig, Kiess, & Fakhry, 2015; Sepiashvili et al., 2015). Not only does this carcinoma affect the US, but it also confers a growing public health concern internationally (Chaturvedi et al., 2011; Elrefaey et al., 2014). Thus, the increasing epidemic of HPV-derived HNSCC is becoming a major health care issue with significant clinical ramifications (Chaturvedi et al., 2011; Elrefaey et al., 2014).

2. High-Risk HPV as an Etiological Factor

HPV infection has been extensively studied in the context of its association with cervical cancer (Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Psyrri et al., 2012), the second leading cancer in women in less developed countries (Adams et al., 2014; Stanley, 2012), and multiple studies have clearly established that HPV infection in the genitals is transmitted by sexual contact (Mannarini et al., 2009; Ramshankar & Krishnamurthy, 2013). The factors responsible for the surge in HPV-derived HNSCC were once nebulous (Mannarini et al., 2009; Wierzbicka et al., 2013), but accumulating evidence now indicates that HPV-initiated OPSCC may be a result of changing sexual behaviors in the population

(Antonsson et al., 2015; Arbyn et al., 2012; Bonilla-Velez et al., 2013; Chaturvedi et al., 2011; D'Souza & Dempsey, 2011; Dalianis, 2014b; Elrefaey et al., 2014; Haedicke & Iftner, 2013; Langer, 2012; Ramshankar & Krishnamurthy, 2013; Wierzbicka et al., 2013). For example, it has been demonstrated that HPV is eight times more likely to be isolated from the oral cavity of sexually experienced individuals than from the oral cavity of those who are sexually inexperienced (Blitzer, Smith, Harris, & Kimple, 2014; Gillison et al., 2012; Nelke, Lysenko, Leszczyszyn, & Gerber, 2013; Rettig et al., 2015). Similarly, oral infection is highly correlated with multiple lifetime sexual partners, early coital debut, oral–genital sex, as well as French kissing (Adams et al., 2014; Blitzer et al., 2014; D'Souza, Agrawal, Halpern, Bodison, & Gillison, 2009; Dalianis, 2014b; Haedicke & Iftner, 2013; Lui & Grandis, 2012; Nelke et al., 2013; Osazuwa-Peters et al., 2015; Wierzbicka et al., 2013). Osazuwa *et al.*, surmised that within the US, a sexually active individual is likely to encounter an HPV infection at one or more points during their lifetime (Moscicki, 2005; Osazuwa-Peters et al., 2015). However, not every HPV infection develops into a carcinoma. In fact, a large majority of infections are transient and clear without any clinical manifestations (Best, Niparko, & Pai, 2012; Bosch et al., 2013; Boscolo-Rizzo et al., 2013; Moscicki, 2005; Psyrri et al., 2012), with 66% of infections clearing within 12 months and 90% within 24 months (Best et al., 2012; Boscolo-Rizzo et al., 2013). Despite the high level of clearance, the presence of high-risk HPV infection in the oral cavity has been associated with a five to fifty-fold increased risk of HNSCC development, depending on the HPV type (Bonilla-Velez et al., 2013; Gillison et al., 2012; Ramshankar & Krishnamurthy, 2013; Wierzbicka et al., 2013). Consequently, the chances of developing

head and neck cancer (HNC) increase when interacting with more than 25 lifetime vaginal sex partners and/or more than five lifetime oral sex partners, according to a study conducted by D'Souza *et al.* (D'Souza et al., 2007; Elrefaey et al., 2014; Pickard, Xiao, Broutian, He, & Gillison, 2012; Ramshankar & Krishnamurthy, 2013). Interestingly, it has been shown that an HPV infection in the head and neck is correlated with an infection in the anogenital area (D'Souza & Dempsey, 2011; Wierzbicka et al., 2013) as cervical cancer patients have a five-fold higher risk of head and neck cancer (Mannarini et al., 2009; Rettig et al., 2015; Steinau et al., 2014). In addition, an increased risk for tongue and tonsil carcinomas are observed in male partners of women with cervical carcinoma (D'Souza & Dempsey, 2011; Elrefaey et al., 2014; Hemminki, Dong, & Frisch, 2000; Rettig et al., 2015), and these results have been corroborated by a match on the HPV type in those couples (Mannarini et al., 2009; Vogt, Gravitt, Martinson, Hoffmann, & D'Souza, 2013; Widdice et al., 2010; Wierzbicka et al., 2013). Therefore, significant accumulated evidence supports the idea that the likely transmission of this infection is primarily through oral–genital and oral–oral routes (Dalianis, 2014b; Mannarini et al., 2009).

Since HPV-positive oropharyngeal cancers display a different etiology than do HPV-negative cancers (Chaturvedi et al., 2011; Sepiashvili et al., 2015; Trosman et al., 2015), HPV-derived OPSCCs are found in a subpopulation of patients that is epidemiologically, genetically, and demographically distinct from patients presenting with the more traditional HPV-negative OPSCCs (Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Gillison et al., 2000; Osazuwa-Peters et al., 2015). Unlike HPV-negative OPSCCs, which are typically found in individuals older than 60 years of age with a strong history of tobacco

and alcohol consumption (T. Cooper et al., 2015; Osazuwa-Peters et al., 2015), HPV-related OPSCC typically appears in younger populations, between the ages of 40 and 55, with generally low levels of substance abuse (Blitzer et al., 2014; Boscolo-Rizzo et al., 2013; Monnier & Simon, 2015; Smith et al., 2004; Wierzbicka et al., 2013). This cohort of patients tends to be high functioning (Bonilla-Velez et al., 2013), and demonstrates a better general condition (Wierzbicka et al., 2013) as well as health (Duek, Billan, Amit, & Gil, 2014; Elrefaey et al., 2014; Friedman et al., 2014; Genden, 2012; Langer, 2012; Lorincz, Jowett, & Knecht, 2015; Nelke et al., 2013; Nichols et al., 2013). Moreover, a recent study reported an 80% higher incidence in males than in females (Dalianis, 2014a; Elrefaey et al., 2014; Garbuglia, 2014; Osazuwa-Peters et al., 2015; Rettig et al., 2015; Sanders, Slade, & Patton, 2012; Zhang, Mirani, Baisre, & Fernandes, 2014) and a lower incidence in blacks than in Caucasians (4% in blacks *vs.* 34% in their Caucasian counterparts) (Chaturvedi et al., 2011; Elrefaey et al., 2014; Rettig et al., 2015; Settle et al., 2009; Weinberger et al., 2010). In addition, this patient cohort possesses higher economic status and more education (Elrefaey et al., 2014; Moore & Mehta, 2015). Therefore, subjects with HPV-related HNSCC are likely to be middle-aged Caucasian males who are non-smokers and non-drinkers with a higher socioeconomic status and educational level (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Rettig et al., 2015).

3. Current Treatments and Therapies

Current therapeutic interventions for HNSCC patients include surgery, chemotherapy, and radiotherapy (Adams et al., 2014; Chai et al., 2015; Duek et al., 2014; Fumagalli et al., 2015). Each of these treatments have been employed at different clinics

in the US (Lui & Grandis, 2012), but currently no clinical guidelines differentiating treatment strategies between HPV-derived and tobacco-derived HNSCC exist (Kaczmar et al., 2014; Mydlarz, Chan, & Richmon, 2015; Psyrri et al., 2012). Moreover, only a few clinical trials have made such a distinction (Ang et al., 2010; Bol & Gregoire, 2014; Elrefaey et al., 2014; Fumagalli et al., 2015; George, 2014; Guo et al., 2015; Lui & Grandis, 2012; Maxwell et al., 2014), even though these two subsets represent separate disease entities pathologically and etiologically (Ang et al., 2010; Dalianis, 2014b; Lui & Grandis, 2012; Trosman et al., 2015; Urban et al., 2014; Zhang et al., 2014). Presently, the standard therapy for head and neck cancer is determined by the tumor stage (Adams et al., 2014; Elrefaey et al., 2014; George, 2014; Machiels et al., 2014), the site of the tumor (Adams et al., 2014; George, 2014; Machiels et al., 2014) and the expected functional outcomes (Machiels et al., 2014), as well as by the preference of the practitioner and the patient, which include considerations of the level of organ preservation and the patient's quality of life (Elrefaey et al., 2014).

Head and neck cancer is classified into the following categories: early-stage or stage I/II, locally advanced or stage III/IV, and recurrent or metastatic phase (Martinez-Useros & Garcia-Foncillas, 2015). Early stages of head and neck cancer are usually treated with a single-modality treatment, such as radiotherapy or surgical resection (Adams et al., 2014; Machiels et al., 2014; Monnier & Simon, 2015; Moore & Mehta, 2015; Purohit, Bhise, Lokanatha, & Govindbabu, 2013). A combination of multiple therapies for superior oncologic results are required for the management of advanced stages III/IV (Kaczmar et al., 2014; Machiels et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015); for example,

surgery with adjuvant radiation or chemoradiation with chemotherapy being added for high risk pathologic features found from the surgical specimen (Bernier et al., 2004; J. S. Cooper et al., 2004; Elrefaey et al., 2014; Haedicke & Iftner, 2013; Sepiashvili et al., 2015), or radiotherapy with concomitant chemotherapy (Burtness, Bourhis, Vermorken, Harrington, & Cohen, 2014; George, 2014; Hamoir et al., 2012; Patel et al., 2015; Sepiashvili et al., 2015). Therefore, patients with advanced stages of head and neck cancer are treated through a multidisciplinary and multimodal treatment approach (T. Cooper et al., 2015; Martinez-Useros & Garcia-Foncillas, 2015; Modur, Thomas-Robbins, & Rao, 2015; Purohit et al., 2013).

3.1. Surgery

Surgery is one of the standard treatments for early stage I/II HNSCC. In the past, surgical procedures sometimes consisted of extensive open transmandibular, and open pharyngotomy procedures (Elrefaey et al., 2014; Ford, Brandwein-Gensler, Carroll, Rosenthal, & Magnuson, 2014; George, 2014; Monnier & Simon, 2015; Mydlarz et al., 2015) that resulted in severe morbidities including facial deformity, dysarthria, and dysphagia (Adams et al., 2014; Duek et al., 2014; Genden, 2012; Mydlarz et al., 2015), especially in more locally advanced cases. Over the past 30 years, advances in radiotherapy and chemotherapy yielding favorable oncologic outcomes shifted treatment choices away from open surgery (Duek et al., 2014; Mydlarz et al., 2015; Nichols et al., 2013), until new minimally invasive trans-oral surgery (TOS) came into prominence as a viable surgical tool for early phase OPSCC (Boscolo-Rizzo et al., 2013; Ford et al., 2014; Lorincz et al., 2015; Maxwell et al., 2014; Mydlarz et al., 2015) within the last decade,

promising to reduce morbidity and mortality while improving organ preservation (Boscolo-Rizzo et al., 2013; Genden, 2012; Urban et al., 2014). This new surgical approach enables resection of a tumor through the opening of the mouth without the damage to normal tissue and musculature seen in transcervical or transmandibular approaches (Mydlarz et al., 2015; Ridge, 2014). Because of these advancements in technology, HPV-associated OPSCC patients may be the most appropriate subgroup to undergo a minimally invasive TOS regimen since they tend to be younger, non-smokers, and have good odds for long-term survival (Boscolo-Rizzo et al., 2013; Mydlarz et al., 2015). Moreover, the restoration of surgical resection as a safe treatment modality reinstituted the advantage of acquiring surgical specimens for definitive pathological staging to guide in the determination of adjuvant therapy needed. Transoral laser microsurgery (TLM) and transoral robotic surgery (TORS) are currently the principal TOS techniques utilized for head and neck carcinoma (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Mydlarz et al., 2015).

TLM is one of the procedures available for early head and neck cancer (Bonilla-Velez et al., 2013). This procedure utilizes surgical apparatus already present in many medical centers, such as a laryngoscope, operating microscope, and a CO_2 laser (Bonilla-Velez et al., 2013; Hinni, Nagel, & Howard, 2015). TLM is capable of conserving normal tissue by resecting the tumors *via* a direct transoral approach using transtumor cuts to assess tumor depth and microscopic magnification to aid in margin control (Bonilla-Velez et al., 2013; Hinni et al., 2015; Lorincz et al., 2015); as a result, the TLM treatment of locally

advanced head and neck cancer can attain excellent cosmetic and functional outcomes (Bonilla-Velez et al., 2013; Genden, 2012).

In 2009, TORS, an alternative method for transoral surgery, became approved for small primary tumors of the head and neck region (Boscolo-Rizzo et al., 2013) and is quickly becoming a popular technique (Ford et al., 2014; Genden, 2012; Lorincz et al., 2015; Mydlarz et al., 2015). TORS's magnified and angled stereoscopic visualization and articulated robotic arms aid in complex resections (Duek et al., 2014; Elrefaey et al., 2014; Genden, 2012; Lorincz et al., 2015) as well as the performance of oncologic extirpations *en bloc* in the oral cavity (Bonilla-Velez et al., 2013; Hinni et al., 2015; Mydlarz et al., 2015). In addition, TORS offers tremor filtration and high-precision motion scaling although at a significantly higher cost (Bonilla-Velez et al., 2013; Duek et al., 2014; Lorincz et al., 2015; Mydlarz et al., 2015). The price for a Da Vinci robotic system surpasses a million dollars, and the additional expenses for services and expendable supplies can be a limiting factor for many clinical centers (Genden, 2012; Lorincz et al., 2015; Mydlarz et al., 2015). However, some of the advantages of the TORS over open surgery include low rates of complications and mortality with shorter postoperative recovery time, as well as satisfactory oncological results and improved swallowing outcomes (Boscolo-Rizzo et al., 2013; Duek et al., 2014; Elrefaey et al., 2014; Genden, 2012). There is some evidence to suggest that TORS resection may allow reduced doses of adjuvant radiation with similar oncologic control and reduced treatment morbidity (Ford et al., 2014; Genden, 2012). To help clarify this, the ECOG 3311 clinical trial is evaluating the de-intensification of postoperative radiation after surgical resection of HPV-associated OPSCC (Kaczmar et al.,

2014; Mydlarz et al., 2015; Ridge, 2014). Therefore, these new trans-oral surgical techniques are decreasing cosmetic disfigurement while improving function and quality of life (Mydlarz et al., 2015; O'Leary & Kjaergaard, 2014).

3.2. Chemotherapy

Cisplatin is the most widely used chemotherapeutic agent with the best prognostic outcome, achieving about a 90% 3-year survival rate (Adams et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015). Cisplatin, also known as cis-Diammineplatinum (II) dichloride or CDDP, is a DNA intercalator targeting cells that replicate at a high rate (Modur et al., 2015). This intercalator binds to guanine residues causing crosslinks between the DNA strands, and eventually leading to cell death (Modur et al., 2015). Studies indicate that HPV-associated patients have a higher response rate to platinum-based chemotherapy than do their HPV-negative counterparts (Modur et al., 2015). However, the benefits of this therapy come at a price due to comorbidities such as, but not limited to, xerostomia, dysphagia, neurotoxicity and renal failure (Adams et al., 2014; Duek et al., 2014; Nichols et al., 2013). This platinum-based regimen continues to be a standard treatment for organ preservation protocols (Adams et al., 2014; Forastiere et al., 2003; Hamoir et al., 2012) as well as advanced and unresectable head and neck cancers (Adams et al., 2014; Psyrri, Seiwert, & Jimeno, 2013). Other commonly used chemotherapeutic agents consist of platinum compounds such as carboplatin; taxanes such as docetaxel and paclitaxel; methotrexate; and 5-fluorouracil (Inhestern et al., 2015; Malhotra et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015). These chemotherapeutic drugs are showing some promise in the treatment of HNSCC patients, however, additional agents that can target

the tumor cells more specifically are under investigation. Targeted chemotherapeutic agents such as cetuximab are discussed below.

3.3. Radiotherapy

Historically, radiotherapy has been thought of as a conventional treatment for HNSCC and is usually a component of a multi-modal therapy plan (Nichols et al., 2013; Vermeer et al., 2013). Radiotherapy induces double strand breaks of the tumor cells, reducing cell viability and increasing cell cycle arrest and death (Hsu et al., 2014). Radiation treatment delivery has evolved through the decades, and advances in radiotherapy have led to the development of intensity-modulated radiotherapy (IMRT) (McBride et al., 2014; Surucu et al., 2015). IMRT delivers radiation to tumor tissues while simultaneously reducing the dosage to non-carcinogenic cells (Broglie et al., 2013; Mydlarz et al., 2015). In this manner, IMRT can more efficiently spare healthy tissues, enhance tumor coverage, and achieve a steady dose distribution (Surucu et al., 2015). Even though IMRT has improved survival outcomes, the toxicities concomitant to irradiation continue to deteriorate a patient's quality of life (Bonilla-Velez et al., 2013; Broglie et al., 2013). For instance, HNSCC treated patients have a higher likelihood of experiencing occlusive carotid artery disease and stroke (Bonilla-Velez et al., 2013; Monnier & Simon, 2015). Moreover, a considerable amount of radiotherapy-induced malignancies become apparent in HNC survivors (Bonilla-Velez et al., 2013). Notwithstanding, the major cause of death in HNC survivors unrelated to cancer is cardiovascular disease associated with radiotherapy (Bonilla-Velez et al., 2013). Since the HPV-dependent OPSCC population is typically younger and exhibits a favorable prognosis, the value of reducing chronic morbidities such as xerostomia

(Genden, 2012; Monnier & Simon, 2015), dysphagia, mucositis, lymphedema, and fibrosis is considerable (Friedman et al., 2014; Genden, 2012; Mydlarz et al., 2015). Therefore, radiation protocols are actively being researched in attempts to decrease both the dosage and duration of therapy (Hinni et al., 2015).

Research has shown that disease control is attainable in both HPV-related and HPV-unrelated subsets when TORS is employed as an initial surgical approach followed by chemoradiation (Boscolo-Rizzo et al., 2013; Haedicke & Iftner, 2013). Unfortunately, these patients are subject to the side effects of surgical procedures as well as those of nonsurgical interventions (Boscolo-Rizzo et al., 2013; Lui & Grandis, 2012). Despite the improvements in therapeutic techniques toward reducing morbidity and increasing survival, the 5-year survival rate of HNSCC patients remains at around 50% (Chai et al., 2015; Coppock et al., 2013; Ford et al., 2014; Haedicke & Iftner, 2013; Inhestern et al., 2015; B. Kumar et al., 2007; Lohaus et al., 2014; Machiels et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015; Zhang et al., 2014).

4. Management of HPV-Associated Tumors: The Debate

Clinicians are becoming increasingly aware of the need for differential therapeutic regimens between HPV-positive and HPV-negative patients (Lui & Grandis, 2012) due to their distinct disease etiologies (Adams et al., 2014; Ang et al., 2010; Gillison et al., 2000; Sepiashvili et al., 2015). Evidence that differences in the biological aspect of these subgroups may affect their prognosis and optimal treatment is increasing (Adams et al., 2014; Bol & Gregoire, 2014; Stern et al., 2012). For example, data collected over the past several years makes a compelling case that patients with HPV-derived OPSCC have a more

favorable survival than do their matched controls, regardless of treatment strategy (Ang et al., 2010; Blitzer et al., 2014; Bol & Gregoire, 2014; Bonilla-Velez et al., 2013; Chaturvedi et al., 2011; Fakhry et al., 2008; Friedman et al., 2014; Fumagalli et al., 2015; Gillison et al., 2000; Guo et al., 2015; Haedicke & Iftner, 2013; Lui & Grandis, 2012; Ramshankar & Krishnamurthy, 2013; Zhang et al., 2014). Research suggests that HPV expression corresponds with increased response rates to conventional chemotherapy (Ang et al., 2010; Arbyn et al., 2012; Bonilla-Velez et al., 2013; Duek et al., 2014; Elrefaey et al., 2014; Fakhry et al., 2008; Wierzbicka et al., 2013; Zhang et al., 2014), radiotherapy (Ang et al., 2010; Antonsson et al., 2015; Arbyn et al., 2012; Bol & Gregoire, 2014; Elrefaey et al., 2014; Gillison et al., 2000; Wierzbicka et al., 2013; Zhang et al., 2014), and radiochemotherapy (RCT) (Ang et al., 2010; Bol & Gregoire, 2014; Bonilla-Velez et al., 2013; Duek et al., 2014; Fakhry et al., 2008; Guo et al., 2015; Lui & Grandis, 2012; Rischin et al., 2010). Moreover, the 3-year overall survival of patients with HPV-associated OPSCC is about 75% as opposed to 50% for those with HPV-unassociated malignancies (Ang et al., 2010; Blitzer et al., 2014; D'Souza & Dempsey, 2011; Lui & Grandis, 2012; Urban et al., 2014; Zhang et al., 2014). Additionally, studies of HPV-positive HNSCC revealed a drop of approximately 50% in recurrences, a 40% decrease in the risk of death (Arbyn et al., 2012; Garbuglia, 2014; Nelke et al., 2013) and a lower incidence of metastases than seen with their HPV-negative counterparts (Blitzer et al., 2014; Elrefaey et al., 2014; Guo et al., 2015; Laskar & Swain, 2015; Psyrri et al., 2012). As impressive as these statistics look, recurrence and metastasis are still responsible for the leading cause of death in HPV-derived OPSCC (Amine et al., 2009; Lui & Grandis, 2012; Trosman et al., 2015). In summary, patients with

HPV-induced tumor report improved therapeutic responses to interventions and better survival rates due to increased sensitivity to chemotherapy and radiotherapy (Adams et al., 2014; Bol & Gregoire, 2014; Bonilla-Velez et al., 2013; Haedicke & Iftner, 2013; Lui & Grandis, 2012; Ramshankar & Krishnamurthy, 2013; Turner et al., 2011).

The reason(s) HPV-related HNSCC are associated with an improved survival outcome as compared to HPV-unrelated cancers remains speculative (Boscolo-Rizzo et al., 2013; Fumagalli et al., 2015; Sepiashvili et al., 2015), but this difference could be ascribed to a variety of factors (Ang et al., 2010; Arbyn et al., 2012). One set of explanations focuses on the patient population, indicating that the favorable prognosis of patients with HPV-associated cancers may be attributable to their younger age at diagnosis (Arbyn et al., 2012; Bol & Gregoire, 2014; Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Modur et al., 2015), their high functioning and superior performance status (Arbyn et al., 2012; Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014), as well as the presence of minimal tobacco and alcohol related co-morbidities (Arbyn et al., 2012; Bol & Gregoire, 2014; Bonilla-Velez et al., 2013; Elrefaey et al., 2014; Modur et al., 2015).

An alternate or possibly complementary explanation focuses on differences in biological mechanisms. That is, even though the biologic mechanisms leading to divergent prognoses in HPV-dependent and independent oropharyngeal cancer have been elusive (Sepiashvili et al., 2015; Zhang et al., 2014), the survival benefit enjoyed by HPV-associated patients could be connected to the molecular differences arising from virus-mediated activities as opposed to events that occur as a consequence of the carcinogens or mutations present in non-HPV cancer patients (Ang et al., 2010; D'Souza et al., 2007;

Genden, 2012). For example, in most tobacco-related tumors, the tumor suppressor gene *TP53* is mutated and inactive, while the *TP53* gene in HPV-infected tumors is wild-type and functionally intact, with the protein being degraded by the HPV oncoprotein E6 (Caicedo-Granados et al., 2014; Elrefaey et al., 2014; Haedicke & Iftner, 2013; van Kempen et al., 2014). Research indicates that persistent treatment with certain therapeutic agents can suppress *E6* oncogenes, allowing the *TP53* gene to carry out its normal function (Genden, 2012; Modur et al., 2015). Therefore, the presence of the wild-type *TP53* gene and the lower mutation rate (Blitzer et al., 2014) observed in HPV-derived SCC may enable these tumor cells to undergo an intact apoptotic response when treated with radiotherapy and/or chemotherapy, resulting in a high response rate (Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Friedman et al., 2014; Genden, 2012; Ramshankar & Krishnamurthy, 2013).

Another possibility is that HPV-positive cancer cells express viral proteins that induce and enhance the immune response, which becomes involved in clearing cancer cells during treatment (Elrefaey et al., 2014; Fertig et al., 2013; Modur et al., 2015; Vermeer et al., 2013). This theory was proposed after a cancer cell line treated with chemoradiotherapy *in vitro* demonstrated increased survival (Friedman et al., 2014) and resistance to treatment (Bol & Gregoire, 2014; Bonilla-Velez et al., 2013) as compared to the same therapy applied *in vivo*, where the cells are surrounded by an immunologic microenvironment. Likewise, an apparent higher response in immunocompetent *vs.* immunodeficient mice further supports this finding (Friedman et al., 2014). In addition, studies indicate that the majority of HPV-infected tumor patients manifest a higher titer

of T cells infiltrating the tumor (Bol & Gregoire, 2014) and a high percentage of cytotoxic $CD8^+$ T cells that are specific to HPV (Blitzer et al., 2014; Bol & Gregoire, 2014; Friedman et al., 2014) compared to non-HPV tumor patients.

Lastly, the difference in the degree of intratumor heterogeneity between HPV-dependent and HPV-independent OPSCC could contribute to their divergent prognoses. Intratumor heterogeneity refers to a tumor population comprised of subpopulations that display differing genetic makeups (Bonilla-Velez et al., 2013). Assuming that certain subpopulations are more susceptible to treatment therapies than others, tumors with high intratumor heterogeneity are progressively identified as having poor therapeutic response and recurrence or metastasis (Bonilla-Velez et al., 2013). HPV-driven tumors are considered to represent a homogeneous, one-agent-induced population, and are thus less intratumorally heterogeneous, possibly leading to the better therapeutic response.

To date, an effective mono-dimensional therapy approach suitable for head and neck carcinoma is not available (Lui & Grandis, 2012). Moreover, the classical therapies generate substantial side effects (Fertig et al., 2013; Hinni et al., 2015). Traditionally, therapeutic strategies have consisted of open surgery with the option of radiochemotherapy (Hinni et al., 2015; Nichols et al., 2013). The adverse effects of these therapeutic interventions have not improved in recent decades, and severe consequences associated with swallowing (Adams et al., 2014; Hinni et al., 2015; Nichols et al., 2013), talking (Adams et al., 2014; Hinni et al., 2015; Nichols et al., 2013), breathing (Hinni et al., 2015), hearing (Adams et al., 2014), and even one's countenance (Adams et al., 2014; Hinni et al., 2015; Nichols et al., 2013) are prevalent. The current contention lies

in whether the intensity level of the therapy is too high for the cohort of HPV-positive patients that exhibit better outcomes (Lui & Grandis, 2012; Nichols et al., 2013; Psyrri et al., 2012; Ramshankar & Krishnamurthy, 2013; Ridge, 2014). The different therapeutic strategies all have comparable oncological effects, yet the functional complications can have a particularly long lasting effect on the rising cohort of young patients with HPV-associated head and neck cancer (Bonilla-Velez et al., 2013; Elrefaey et al., 2014). In making their decisions, clinicians are dealing with a subset of patients that will most likely reach full recovery and surpass their cancer by a few decades, and hence will be severely affected by the late sequelae of cancer treatment (Bonilla-Velez et al., 2013; Duek et al., 2014; Elrefaey et al., 2014; Laskar & Swain, 2015; Lorincz et al., 2015). Consequently, an intensive multidisciplinary regimen resulting in considerable morbidity might be inappropriate for the HPV-initiated HNSCC subgroup (Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014). Accordingly, the favorable prognosis in HPV-driven oropharyngeal cancer has prompted the progression to organ preservation strategies (Bonilla-Velez et al., 2013; Nichols et al., 2013; Psyrri et al., 2012) that treat the tumor with minimal cosmetic and functional complications (Dalianis, 2014a). Therefore, evaluating the options for therapeutic de-escalation to reduce toxicity and determining treatment strategy with high efficacy to optimize quality of life is of utmost importance for this HPV-associated subpopulation (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Coppock et al., 2013; George, 2014; Lui & Grandis, 2012; Masterson et al., 2014; Nichols et al., 2013).

Some researchers contend that concurrent radiochemotherapy may confer excess treatment (Boscolo-Rizzo et al., 2013). Moreover, evidence has surfaced denoting the

overtreatment of adjuvant chemotherapy after surgical resection in locally advanced HNSCC patients (Hinni et al., 2015), accruing proponents for the de-escalation regimens. Yet the establishment of a de-intensification regimen can be challenging since nearly 10% of patients with HPV-derived tumors have a poorer prognosis and a higher likelihood of developing metastases or recurrence (Ang et al., 2010; Boscolo-Rizzo et al., 2013; Lui & Grandis, 2012; Sepiashvili et al., 2015), demanding a more potent therapy. Some advise not to change treatment decisions or management strategy on the basis of HPV, as conclusive evidence is lacking (Blitzer et al., 2014; Bosch et al., 2013; Machiels et al., 2014; Masterson et al., 2014; Ramshankar & Krishnamurthy, 2013; Urban et al., 2014). Others argue that the treatment of patients with HPV-associated OPSCC should depend on the tumor phase (Urban et al., 2014), the general condition and performance status of the patient, and the expected functional outcomes (Boscolo-Rizzo et al., 2013). Their aim is to increase the opportunities to tackle early phase carcinomas with a mono-dimensional regimen (Boscolo-Rizzo et al., 2013). Further investigation is necessary to determine whether an alternative treatment strategy is required for HPV-associated HNC patients.

5. De-Intensification Trials

Clinical trials testing various de-intensification strategies for HPV-positive head and neck carcinoma patients are under examination (Bonilla-Velez et al., 2013; Psyrri et al., 2012). The de-escalation of therapy intensity may be achieved through several different approaches (Duek et al., 2014; Langer, 2012). An initial proposal was to decrease the standard dose of definitive radiotherapy or chemoradiotherapy, since radiation is considered the most toxic component of a therapeutic regimen (Bonilla-Velez et al., 2013;

Psyrri et al., 2012). An Eastern Cooperative Oncology Group (ECOG1308) phase II trial evaluated the response to chemotherapy with paclitaxel, carboplatin, and cetuximab, and based on their complete response, determined which patients could safely undergo radiation dose reduction (Genden, 2012; Laskar & Swain, 2015; Lui & Grandis, 2012; Psyrri et al., 2012; Psyrri et al., 2013). In 2014, the investigators revealed positive initial results in patients that underwent the dose reduction (Friedman et al., 2014).

Another strategy is to employ the new minimally invasive TOS technique as a primary surgical therapy (Bonilla-Velez et al., 2013; Duek et al., 2014). A randomized trial, ECOG3311, evaluating whether initial transoral surgery (TORS) can allow for decreased adjuvant dose radiotherapy for patients with HPV-positive HNC is currently in progress in the US (Blitzer et al., 2014; Bonilla-Velez et al., 2013; Laskar & Swain, 2015; Ridge, 2014).

Another possibility is the administration of a less toxic alternate agent, such as cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody (Duek et al., 2014). The Radiation Therapy Oncology Group study (RTOG 1016) and De-ESCALaTE phase III trials are comparing conventional cisplatin concurrently with radiotherapy to the new cetuximab with concomitant radiation in HPV-driven locally advanced oropharyngeal squamous cell carcinoma (SCC) (Adams et al., 2014; Blitzer et al., 2014; Bonilla-Velez et al., 2013; Langer, 2012; Laskar & Swain, 2015; Lui & Grandis, 2012; Psyrri et al., 2012).

6. Molecular Mechanisms

Ever since the presence of HPV was demonstrated in tissues of HNSCC patients in 1983, the study of molecular mechanisms in HPV-associated HNSCC has garnered significant attention (Adams et al., 2014; Friedman et al., 2014; Ramshankar &

Krishnamurthy, 2013; Syrjanen, Pyrhonen, Syrjanen, & Lamberg, 1983). Insight accumulated on the molecular progression of HPV derives from the extensive research performed on cervical tumorigenesis (Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Modur et al., 2015; Psyrri et al., 2012); consequently, cervical cancer has become the standard model for HPV studies (Adams et al., 2014; Bosch et al., 2013). With an epidemic on the horizon, it will be vital to adjust our understanding of the properties of HPV in cervical carcinoma to be applicable to head and neck carcinoma (Adams et al., 2014).

Approaches already developed for the treatment and prevention of cervical cancer may be of great help in combating HPV-derived HNSCC (Adams et al., 2014). Nonetheless, the different anatomical and molecular aspects between cervical and oropharyngeal carcinoma must be delineated to adapt the current knowledge to the oral context (Adams et al., 2014). For example, estrogen signaling plays a significant role in cervical cancer, while hormonal dependence is not discernible in head and neck carcinomas (Adams et al., 2014; Langevin, Grandis, & Taioli, 2011). Furthermore, the cervix is not as frequently exposed to elevated amounts of cytotoxic agents and chemical carcinogens as the oropharynx (Boscolo-Rizzo et al., 2013). The distribution of specific HPV types detected in the two cancers varies as well, revealing a broad spectrum of high-risk HPV types accounting for cervical cancer in comparison to the more limited variety observed in head and neck carcinomas (Adams et al., 2014). Another difference observed is that, contrary to the integrated HPV form predominant in cervical cancers (Jeon, Allen-Hoffmann, & Lambert, 1995; Yu et al., 2005), the HPV genome in HNSCC samples is frequently found in both episomal and integrated forms (Gao et al., 2014; Koskinen et al., 2003; Mannarini et

al., 2009; Olthof et al., 2015; Ramshankar & Krishnamurthy, 2013; Rettig et al., 2015), indicating that integration is not essential for progression of tumorigenesis in this location (Adams et al., 2014; Mannarini et al., 2009). Additionally, the presence of HPV in different cancers engenders divergent prognoses (Zhang et al., 2014). That is, while HPV-driven HNSCC have better treatment outcomes, the presence of HPV in cervical cancer is associated with poor prognosis (Albers et al., 2005; Zhang et al., 2014), and HPV-associated cervical cancers are considered more chemoresistant than are other gynecological tumors (Rein & Kurbacher, 2001). These differential prognoses may be due to the distinctive properties and elements characteristic of the host cancer that come into play with the virus, and might contribute substantially to the pathogenesis of HPV malignancy (Zhang et al., 2014). Despite these considerations, the molecular virology of infection is not anticipated to be significantly different in HNSCC as compared to that present in cervical cancer. The prevailing understanding of the molecular details of HPV has therefore shed light on HPV-positive head and neck cancer.

HPV is transmitted through the mucosal and non-mucosal skin epithelia (Adams et al., 2014; Blitzer et al., 2014). About 200 HPV types categorized based on the HPV L1 sequence have been detected, some of which have the ability to induce carcinogenesis (Adams et al., 2014; Blitzer et al., 2014; S. Kumar, Biswas, & Jose, 2015; Psyrri et al., 2012; Stanley, 2012). Nearly 40 of these HPV types affect the mucosal tissues (S. Kumar et al., 2015) and can be stratified into low-risk (HPV 6,11) and high-risk (*i.e.*, HPV-16, 18) categories, based on their ability to develop precancerous lesions and their potential to cause malignant transformation (Adams et al., 2014; Blitzer et al., 2014; Bol & Gregoire,

2014; Bonilla-Velez et al., 2013; Cubie & Cuschieri, 2013; Nicol et al., 2013; Stanley, 2012). The oncogenic high-risk subtypes are expected to give rise to 5.2% (Adams et al., 2014; Bonilla-Velez et al., 2013; Bosch et al., 2013; Parkin, 2006; Sepiashvili et al., 2015) of cancers globally, being responsible for up to 70% of oropharyngeal (Sepiashvili et al., 2015; Stanley, 2012), 99% of cervical (Sepiashvili et al., 2015), 88% of anal (Sepiashvili et al., 2015), and 70% of vaginal (Elrefaey et al., 2014; Sepiashvili et al., 2015; Stanley, 2012) lesions. Of the 20 identified carcinogenic high-risk HPV types (Blitzer et al., 2014; S. Kumar et al., 2015), HPV-16 is the most rampant (Garbuglia, 2014; Nelke et al., 2013), accounting for more than 90% of HPV-positive oropharyngeal cancers (Adams et al., 2014; Antonsson et al., 2015; Bol & Gregoire, 2014; Sepiashvili et al., 2015), followed by HPV-18 (Osazuwa-Peters et al., 2015).

The HPV is a non-enveloped, double-stranded DNA virus that displays a predilection for squamous cell epithelium (Adams et al., 2014; Blitzer et al., 2014; Bonilla-Velez et al., 2013; Lin, Doolan, Hung, & Wu, 2010; Stanley, 2012). The stratified squamous epithelium is composed of progenitor cells in the lower *stratum*, and as they move up the suprabasal layer (Blitzer et al., 2014; Ramshankar & Krishnamurthy, 2013), they become differentiating keratinocytes (Adams et al., 2014; Modur et al., 2015). HPV infection occurs when small lesions or tears at the surface of the epithelium are present, granting the virus entry to the progenitor cells in the basal layer of the stratified epithelium (Adams et al., 2014; Blitzer et al., 2014; Modur et al., 2015; Ramshankar & Krishnamurthy, 2013). Following an infection, the virus will seize the host cellular machinery to synthesize viral nucleic acids and transcribe proteins, though usually at low levels (Adams et al., 2014;

Best et al., 2012; Boscolo-Rizzo et al., 2013). HPV then takes advantage of the differentiation process in these keratinocytes to complete its life cycle (Adams et al., 2014; Best et al., 2012; D'Abramo & Archambault, 2011). When the differentiating cells reach the top *stratum* of the epithelium, HPV will proceed with protein coat formation, assembly of the new viral components, and eventual viral release (Adams et al., 2014). Though the process described does not normally lead to cancer, certain events can trigger HPV to transform the differentiating keratinocytes into SCC (Boscolo-Rizzo et al., 2013).

The HPV genome is composed of approximately 8,000 base pairs (Nicol et al., 2013) with dual promoters that encode two separate groups of viral proteins (Blioumi et al., 2014; Bol & Gregoire, 2014; S. Kumar et al., 2015; Lin et al., 2010). The non-structural or early genes *E1*, *E2*, *E4*, *E5*, *E6*, and *E7* are involved in viral replication, and the structural or late genes *L1* and *L2* control the viral packaging (Adams et al., 2014; S. Kumar et al., 2015; Lin et al., 2010; Stanley, 2012). E1 manages the replication and transcription of the virus by acting as a DNA helicase (Adams et al., 2014), and is the only viral protein with enzymatic activity (Stanley, 2012). E2 can regulate the HPV genome and down-regulate the expression of E6 and E7 oncoproteins by binding to their promoters (Adams et al., 2014; Lin et al., 2010). The activity of E4 is less well understood, but findings suggest that its interactions with the intermediate filaments of the keratin cytoskeleton may assist with viral release (Adams et al., 2014; Almajhdi, Senger, Amer, Gissmann, & Ohlschlager, 2014).

The immortalizing qualities of the virus are attributable primarily to the oncoproteins E6 and E7 (Adams et al., 2014; Bol & Gregoire, 2014; D'Abramo & Archambault, 2011;

Elrefaey et al., 2014) with additional contributions from E5 (Blitzer et al., 2014). The cooperation between these three oncoproteins and with their interacting cellular partners promotes the transformation of the host's epithelium and maintenance of the phenotype that leads to tumorigenesis (Adams et al., 2014; Best et al., 2012; Blitzer et al., 2014; Bol & Gregoire, 2014; D'Abramo & Archambault, 2011; Psyrri et al., 2012). As currently understood, the function of E5 is to subvert immune surveillance by repressing the *major histocompatibility complex* (MHC) class I molecules in the host cells (Best et al., 2012; Yuan, Filippova, & Duerksen-Hughes, 2012). Moreover, the E5 oncoprotein, particularly E5 from HPV-16, is involved with trafficking and signaling through the EGFR pathway (Best et al., 2012; Yuan, Filippova, & Duerksen-Hughes, 2012).

The oncoproteins E6 and E7 are constitutively expressed throughout the progression of the carcinoma (Stern et al., 2012), making them attractive targets for antiviral therapy (Almajhdi et al., 2014; D'Abramo & Archambault, 2011; Devaraj, Gillison, & Wu, 2003; Grasso et al., 2013; Ma et al., 2012; Monie, Tsen, Hung, & Wu, 2009). In the case of cervical cancer, the elevated expression of the E6 and E7 oncoproteins is attributed to the integration of HPV into the genome of the host, in such a way as to deregulate expression of the negative regulator E2 (Adams et al., 2014; Dalianis, 2014a; Gao et al., 2014; Lin et al., 2010). However, integration seems to be less necessary for the development of HNSCC, indicating that the enhanced expression of viral oncogenes in this context can be independent of viral integration (Olthof et al., 2015; Psyrri et al., 2012). We can speculate that the reason for the expression of oncoproteins in episomal HPV oral cancer may be

exposure to exogenously derived factors, which can synergistically work in conjunction with the virus to elicit tumorigenesis.

The central role of the oncogenic protein E6 is to inhibit apoptosis of the infected cells by accelerating the degradation of apoptotic mediators, including the well-known tumor suppressor protein p53 (Cherry et al., 2013; M. S. Lechner & Laimins, 1994; Nicol et al., 2013; Scheffner, Werness, Huibregtse, Levine, & Howley, 1990), thereby removing these proteins from functioning in the intrinsic apoptotic pathway (Yuan, Filippova, & Duerksen-Hughes, 2012). The HPV E6 oncoprotein induces ubiquitination of p53 by complexing with E6AP, an E3 ubiquitin ligase (Kennedy et al., 2014; Ramshankar & Krishnamurthy, 2013). The resulting annihilation of p53 leads to the prevention of cell cycle arrest and/or apoptosis (Caicedo-Granados et al., 2014; D'Abramo & Archambault, 2011; Kennedy et al., 2014; Ramshankar & Krishnamurthy, 2013). E6 proteins from high risk and low risk HPV types are both able to bind to p53, however, only the high-risk types are able to carry it through to proteasomal degradation (D'Abramo & Archambault, 2011; Hietanen, Lain, Krausz, Blattner, & Lane, 2000). In addition to blocking the intrinsic apoptotic pathway through p53 degradation, E6 is able to protect host cells from extrinsic apoptosis, which is triggered by the binding of *tumor necrosis factors* (TNF)-family ligands to their corresponding receptors (Yuan, Filippova, & Duerksen-Hughes, 2012). For example, E6 has been shown to bind to major players of the extrinsic apoptotic pathway such as the initiator of the caspase cascade, procaspase 8 (Filippova et al., 2007; Tungteakkhun, Filippova, Fodor, & Duerksen-Hughes, 2010), as well as the adaptor molecule Fas-associated Death Domain (FADD) (Filippova, Parkhurst, & Duerksen-

Hughes, 2004; Tungteakkhun, Filippova, Neidigh, Fodor, & Duerksen-Hughes, 2008). E6 binding to these substrates leads to their accelerated degradation, thereby inhibiting the transmission of apoptotic signals to effector caspases such as caspases 3 and 7. As a result, E6 prevents cells from undergoing apoptosis initiated through both the intrinsic and extrinsic pathways (Tungteakkhun & Duerksen-Hughes, 2008).

Another oncogene, *E7*, enhances cellular proliferation by inactivating the retinoblastoma *protein (*pRb) and other proteins involved in the control of cell division (Boyer, Wazer, & Band, 1996; Cherry et al., 2013; Dyson, Howley, Munger, & Harlow, 1989; Elrefaey et al., 2014; Garbuglia, 2014; Nicol et al., 2013). The HPV E7 protein binds to the pRb-E2F complex and removes pRb from the complex, leading to the disruption of cell cycle controls (Kennedy et al., 2014; X. Liu, Clements, Zhao, & Marmorstein, 2006; Ramshankar & Krishnamurthy, 2013). Hence, a therapeutic strategy that targets these oncogenes would target the cells that have been infected and transformed by reactivating their intrinsic and extrinsic apoptotic pathways and regaining cell cycle control. Such promising avenues could potentially augment the effectiveness of current modalities while reducing toxicity and morbidities.

7. HPV Detection and Screening Tools

The majority of head and neck carcinomas are discovered at late stages of tumor progression, arguing for the need of a reliable detection tool that is clinically relevant to facilitate early detection of HNSCC (Adams et al., 2014; Dreyer et al., 2013). Considering factors of age, stage of disease, and tobacco smoking status in these cancer patients, the most significant prognostic indicator of survival found to date is HPV status (Ang et al.,

2010; Dalianis, 2014a; Elrefaey et al., 2014; Friedman et al., 2014; George, 2014; Machiels et al., 2014; Modur et al., 2015; Ramshankar & Krishnamurthy, 2013; Vermeer et al., 2013). It is estimated that HPV affects approximately 70% of all carcinomas in the oropharynx and the oral cavity (Chaturvedi et al., 2011; D'Souza & Dempsey, 2011; Elrefaey et al., 2014; Haedicke & Iftner, 2013; Lui & Grandis, 2012; Mannarini et al., 2009; Nelke et al., 2013; Patel et al., 2015; Surucu et al., 2015). Moreover, since HPV-related OPSCC has a remarkably more favorable prognosis than does HPV-unrelated cancer (Haedicke & Iftner, 2013), establishing HPV status through an effective screening tool will offer significant advantages.

In contrast to the case with cervical cancer, there are no reliable screening methods or routine check-ups equivalent to the Pap smear to detect early HPV neoplasia in the oral cavity (Adams et al., 2014; Haedicke & Iftner, 2013; Moore & Mehta, 2015; Wierzbicka et al., 2013). Moreover, since the infected tissue in the oral cavity normally arises in an inaccessible location, devising and implementing such a tool for regular diagnosis becomes challenging (Adams et al., 2014; Kreimer, 2014; Rettig et al., 2015), leaving it up to the patients to consistently monitor for symptoms such as continual sore throats, oral lesions, or swollen masses or glands (Adams et al., 2014; Moore & Mehta, 2015). Unfortunately, these relatively mild and non-alarming manifestations tend to go unnoticed quite frequently, compounding the issue that most head and neck carcinomas are identified at later tumor stages by the time of diagnosis (Adams et al., 2014; Chai et al., 2015; Hsu et al., 2014; Inhestern et al., 2015; John et al., 2013). Consequently, finding

accurate and practical methods to assess the presence of HPV in the oral cavity is a high priority (Elrefaey et al., 2014).

At this point, the technique(s) to be employed for determining the HPV status of head and neck cancers is controversial, due to variations in available methods in terms of cost, sensitivity, technicality, specificity, and reliability (Bishop, Lewis, Rocco, & Faquin, 2015; Bonilla-Velez et al., 2013; Bosch et al., 2013; Dreyer et al., 2013; Elrefaey et al., 2014; Jarboe, Hunt, & Layfield, 2012; Linxweiler et al., 2015; Ramshankar & Krishnamurthy, 2013; Wierzbicka et al., 2013). Three common methods of detection are currently used: Polymerase Chain Reaction (PCR), in situ hybridization (ISH), and p16 immunohistochemistry (IHC) (Elrefaey et al., 2014; Lorincz et al., 2015). In particular, the detection of the viral DNA, such as *E6* or *E7* sequences (Dictor & Warenholt, 2011; Weiss, Heinkele, & Rudack, 2015) through PCR or ISH has been a very common practice (Boscolo-Rizzo et al., 2013; Melkane et al., 2014; Wittekindt, Wagner, & Klussmann, 2011). PCR is highly sensitive, detecting as little viral DNA as 0.001 copy per genome from tumor samples, plasma or salivary collections (Ahn et al., 2014; Bonilla-Velez et al., 2013). It can also assess the viral load (Jarboe et al., 2012) and identify the viral subtype by probing for the *L1* region of the HPV genome (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Jarboe et al., 2012; Kelesidis et al., 2011; Weiss et al., 2015). A disadvantage of focusing on the *L1* region is that this region can be compromised or deleted following integration into the host genome (Kimple, Torres, Yang, & Kimple, 2012; Weiss et al., 2015), thereby leading to underestimates of the presence or the viral load of HPV (Gillison et al., 2000). Furthermore, since PCR detects a region of the viral genome indiscriminately of whether

it is in the integrated or episomal form, this method does not have the ability to determine the physical status of the virus nor its activity, which are essential in assessing tumor development (Bonilla-Velez et al., 2013; Melkane et al., 2014; Weiss et al., 2015). Additionally, this method is rather expensive and is therefore only utilized in select laboratory centers (Bonilla-Velez et al., 2013; Duncan et al., 2013). On the other hand, ISH is highly specific in detecting viral integration status and transcriptional activity (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Melkane et al., 2014). It utilizes a fluorescent-labeled probe to localize and visualize the HPV DNA in the host genome of the tumor dissection (Jarboe et al., 2012; Weiss et al., 2015). Diffuse signals indicate the presence of episomal HPV, while punctate signals represent the integrated forms (Smeets et al., 2007). Nevertheless, since ISH does not amplify the viral genome, this method is not as sensitive (Weiss et al., 2015) or as fast as PCR. However, the procedure can be automated and has become available in certain clinical laboratories (Bonilla-Velez et al., 2013; Jarboe et al., 2012).

The detection of HPV E6/E7 mRNA is the “gold standard” validation of active HPV oncoprotein transcription, and is considered clinically applicable in the evaluation of carcinogenesis (Bishop et al., 2012; Boscolo-Rizzo et al., 2013; Dreyer et al., 2013; Melkane et al., 2014). Since mRNA is very fragile and easily degraded, fresh or rapidly frozen samples are required for this approach (Boscolo-Rizzo et al., 2013; Melkane et al., 2014). While the detection of mRNA through reverse-transcriptase PCR or RT-PCR is technically challenging and perceived as inappropriate for routine screening (Boscolo-Rizzo et al., 2013), the novel ISH assay, RNAscope, has been met with great interest and

found to be perhaps the most promising of available methods (Bishop et al., 2012; Melkane et al., 2014).

Another major alternative for detecting the virus is the IHC of the CDK inhibitor p16, a transcript encoded by the *CDKN2A* gene (Boscolo-Rizzo et al., 2013; Lorincz et al., 2015; Weiss et al., 2015). This technique has become popular due to its high sensitivity (Bonilla-Velez et al., 2013), technical ease, swiftness, practicality (Blitzer et al., 2014; Bonilla-Velez et al., 2013; Duncan et al., 2013; Melkane et al., 2014), inexpensiveness (Blitzer et al., 2014; Bonilla-Velez et al., 2013; Duncan et al., 2013; Melkane et al., 2014), and adequate consistency with PCR and ISH (Bonilla-Velez et al., 2013). p16 is considered a suitable surrogate marker of HPV infection (Boscolo-Rizzo et al., 2013; Ramshankar & Krishnamurthy, 2013), and is biologically relevant because its overexpression corresponds closely to the transformation of infected cells (Adams et al., 2014; Weiss et al., 2015). p16 becomes up-regulated when E2F is released from the E2F-pRb complex after pRb is degraded by E7 (Adams et al., 2014; Blitzer et al., 2014; Boscolo-Rizzo et al., 2013; Fertig et al., 2013; Ramshankar & Krishnamurthy, 2013). This method of detection is the most widespread across multiple clinical centers (Blitzer et al., 2014; Melkane et al., 2014). It should, however, be noted that not all tumors that test positive for p16 contain HPV (Blitzer et al., 2014). Across various tests, HPV infection has not been identified in approximately 10%–20% of $p16^{+}$ head and neck carcinomas (Blitzer et al., 2014; Melkane et al., 2014). Since the practice, interpretation, and reporting of p16 IHC differ, in some cases its prognostic diagnosis can be misinformative and hence unreliable as a stand-

alone method (Bonilla-Velez et al., 2013; Boscolo-Rizzo et al., 2013; Elrefaey et al., 2014; Melkane et al., 2014).

Many investigators propose that using RT-PCR to detect the presence of E6/E7 mRNA may be suitable as a gold standard for fresh samples, since the expression of these two oncogenes is characteristic of a functional HPV infection and cell transformation (Arbyn et al., 2012; Boscolo-Rizzo et al., 2013; Dalianis, 2014a; Dreyer et al., 2013). However, this method requires further examination (Melkane et al., 2014). According to one study, the employment of HPV-PCR or p16 IHC alone is not very reliable or clinically adequate (Fonmarty et al., 2015); notwithstanding, Dalianis *et al.* reported that a HPV DNA test such as PCR in addition to an evaluation of p16 overexpression through IHC is regarded as "specific and sensitive as utilizing a gold standard" (Arbyn et al., 2012; Boscolo-Rizzo et al., 2013; Dalianis, 2014a; Shi et al., 2009; Smeets et al., 2007). Yet another panel of experts has suggested a "cost-efficient" stepwise algorithm to reliably determine HPV infections, which includes an initial testing of p16 through IHC followed by an HPV ISH to confirm the IHC results (Bonilla-Velez et al., 2013; Melkane et al., 2014). If the tests provide conflicting results, a PCR or an ISH probe for specific HPV types can be utilized (Bonilla-Velez et al., 2013). This sequence of methods is thought to provide the highest specificity for determining HPV status (Melkane et al., 2014; Ramshankar & Krishnamurthy, 2013). Others have suggested variations of these detection methods and proposed a variety of combinations (Boscolo-Rizzo et al., 2013; Linxweiler et al., 2015; Melkane et al., 2014). In order to standardize the detection methods in clinical settings and to design reliable clinical research, a unanimous agreement on the most reliable

detection tool(s) for HPV status is required and requisite (Bonilla-Velez et al., 2013; Dreyer et al., 2013).

8. Prophylactic Vaccines

A steep upward shift in the incidence of HPV-derived HNSCC demands a search for a vaccine that can avert the infection of oral HPV before an opportunity to develop a malignant lesion arises, especially considering the lack of a reliable routine screening tool for those at risk of oropharyngeal SCC (Adams et al., 2014; Chaturvedi et al., 2011). Past vaccines have been effective at immunizing against viruses such as influenza and varicella, and such prototypes should help in the development of prophylactics against oral HPV infection (Adams et al., 2014).

Preventive vaccines against HPV in the cervix have been developed and have become available to the public within the past decade (Boscolo-Rizzo et al., 2013). The first prophylactic vaccine to be approved was Gardasil, a quadrivalent vaccine that prevents infection from high-risk HPV types 16 and 18 as well as the low-risk HPV types 6 and 11 (Adams et al., 2014; Best et al., 2012). Cervarix has been developed as a bivalent vaccine that immunizes against HPV types 16 and 18 (Adams et al., 2014; Best et al., 2012). Both prophylaxes encompass the predominant high-risk HPV types that are found in cervical malignancy, whereas the quadrivalent vaccine also targets genital warts and contains in addition the two most prevalent non-oncogenic viral types (Adams et al., 2014; Bonilla-Velez et al., 2013). Despite the fact that Cervarix excludes the low-risk HPV types, a study that compares both prophylaxes indicated that Cervarix is able to produce a stronger antibody response than Gardasil against the two oncogenic HPV types (Best et al., 2012).

Phase III trials of these vaccines established efficacy and safety in the protection against anogenital HPV infections, lesions, and warts, but these prophylaxes have not been certified for the immunization of HPV infection in the head and neck region (Adams et al., 2014; Boscolo-Rizzo et al., 2013). Notwithstanding, there is great potential that the current HPV vaccination will prevent oral HPV infection (Boscolo-Rizzo et al., 2013; Dalianis, 2014a). A trial that was originally intended to examine the efficacy of the HPV vaccine in cervical infections has collected oral rinses that showed encouraging results of the vaccine's effectiveness in obviating HPV infection from the oral cavity (Kreimer, 2014; Moore & Mehta, 2015; Osazuwa-Peters et al., 2015; Rettig et al., 2015).

In contrast to the large diversity of high-risk HPV types observed in cervical carcinoma (Adams et al., 2014), HPV types 16 and 18 constitute over 95% of HPV-positive tonsillar and oropharyngeal cancers (Dalianis, 2014a; Haedicke & Iftner, 2013; Osazuwa-Peters et al., 2015). Hence, the current prophylactic vaccines can be highly effective at preventing HPV-derived HNSCC, since they encompass the primary HPV types that are causal of OPSCC (Adams et al., 2014). Moreover, although clinical evidence supporting their efficacy in the prevention of head and neck cancers is not yet documented (Boscolo-Rizzo et al., 2013; Haedicke & Iftner, 2013), these vaccines have demonstrated that they can induce a systemic robust humoral response against the oncogenic HPV types 16 and 18, and hence should in principle be efficacious against oral infections (Adams et al., 2014; Boscolo-Rizzo et al., 2013; Ramshankar & Krishnamurthy, 2013). Ongoing clinical trials are currently assessing the effectiveness of the quadrivalent HPV vaccine against HPV infection in the oral cavity (Mannarini et al., 2009). The effect of these prophylactic HPV

vaccines on oropharyngeal HPV infection and HPV-derived head and neck cancer will be clearer once further results are obtained (Best et al., 2012; Haedicke & Iftner, 2013; Rettig et al., 2015).

9. Therapeutic Vaccines

Therapeutic vaccines for HPV-driven malignancies are currently undergoing clinical investigations (Psyrri et al., 2012; Ramshankar & Krishnamurthy, 2013). Unlike the previously described prophylactic vaccines, which offer no protection against individuals already infected with HPV (Cherry et al., 2013; D'Abramo & Archambault, 2011; Devaraj et al., 2003; Elrefaey et al., 2014; Haedicke & Iftner, 2013), therapeutic vaccines are intended to treat the individual by eliciting a cell-mediated response that can recognize and attack an established dysplasia or persistent infection (S. Kumar et al., 2015; Mannarini et al., 2009; Psyrri et al., 2012). Moreover, in contrast to prophylactic vaccines, which incite an antibody-mediated humoral response to clear the virus and to prevent access to the squamous epithelium, therapeutic vaccines must activate the T cell-mediated immune system to destroy the existing HPV-infected cells and prevent them from developing into carcinomas (Best et al., 2012; Lin et al., 2010; Ma et al., 2012). This can be challenging for immunocompromised patients because of their weakened immune system; hence, these vaccines are anticipated to be most effective in immunocompetent individuals.

In the design and development of therapeutic vaccines, HPV-16 E6 and E7 oncoproteins have become popular viral targets since they are consistently expressed in HPV malignancies and are critical for transformation (Devaraj et al., 2003; Grasso et al., 2013; S. Kumar et al., 2015; Ma et al., 2012; Monie et al., 2009; Psyrri et al., 2012; Stern

et al., 2012). Moreover, in contrast to tumorigenic antigens derived from mutated or overexpressed self-proteins, viral E6 and E7 are entirely foreign proteins, which express numerous antigenic epitopes and thus contribute toward an enhanced immune response (Devaraj et al., 2003; Monie et al., 2009; Psyrri et al., 2012). More importantly, only the infected cells will express these viral proteins, making them ideal targets for therapy of HPV-derived cancers (Ma et al., 2012; Psyrri et al., 2012). A majority of clinical trials for therapeutic vaccines are in their early phase and have focused on feasibility, immunogenicity, and safety (Almajhdi et al., 2014; Ramshankar & Krishnamurthy, 2013). Multiple vaccines are currently being explored as potential therapeutic strategies including DNA vaccines, peptide and protein vaccines, cell-based vaccines, as well as bacterial and viral live vector vaccines (Devaraj et al., 2003; Grasso et al., 2013; S. Kumar et al., 2015; Ma et al., 2012; Psyrri et al., 2012; Ramshankar & Krishnamurthy, 2013).

Due to their safety, ease of production, purity and stability, DNA vaccines have become attractive therapeutic candidates for HPV-associated HNSCC (Devaraj et al., 2003; S. Kumar et al., 2015; Lin et al., 2010; Ma et al., 2012; Monie et al., 2009; Psyrri et al., 2012). DNA vaccines introduce plasmid DNA into the host and promote its transcription and immune presentation of the encoded HPV proteins by the transfected cells (S. Kumar et al., 2015; Ma et al., 2012; Monie et al., 2009). This MHC presentation elicits T cell-mediated and/or antibody-mediated responses that attack the encoded antigen (S. Kumar et al., 2015; Ma et al., 2012; Monie et al., 2009). However, DNA vaccines can have low immunogenicity because they lack the ability to spread the DNA from the transfected cells and amplify it in the neighboring cells (Lin et al., 2010; Monie et al.,

2009). Despite such limitations, significant results from the therapeutic HPV DNA vaccine studies have progressed to various clinical investigations (Monie et al., 2009). For example, a phase I trial at Johns Hopkins University is evaluating a DNA vaccine targeting HPV-16 E7 antigens in patients with advanced HPV-16-positive OPSCC (Mirghani et al., 2015; Monie et al., 2009; Psyrri et al., 2012). This vaccine encodes for HPV-16 E7 fused to the immuno-modulatory agent calreticulin, a protein that can stimulate natural killer T cells and enhance MHC class I antigen presentation (Grasso et al., 2013; Mirghani et al., 2015; Monie et al., 2009; Psyrri et al., 2012).

In contrast, peptide vaccines are taken up by antigen presenting cells (APC) directly without the need for encoding and are loaded onto MHC molecules for antigenic presentation (S. Kumar et al., 2015; Psyrri et al., 2012). This leads to activation of an antigen specific T cell response and putative elimination of infected cells (S. Kumar et al., 2015). Peptide vaccines are safe, stable, and easily prepared, but have poor immunogenicity (S. Kumar et al., 2015; Lin et al., 2010; Monie et al., 2009). Some adjuvants used to circumvent the low immunogenicity include costimulatory molecules, cytokines, chemokines, and Toll-like receptor (TLR) ligands (Lin et al., 2010; Monie et al., 2009). Specific examples include calreticulin, Montanide ISA-51, and GM-CSF, (Elrefaey et al., 2014; Grasso et al., 2013; Lin et al., 2010; Psyrri et al., 2012). Another disadvantage with respect to peptide vaccines is that they are MHC restricted, which limits their widespread use (Lin et al., 2010; Monie et al., 2009). However, this restriction can be overcome by the use of overlapping long peptides that harbor several epitopes of the antigen (Lin et al., 2010). One study has devised an HPV peptide vaccine composed of

synthetic long overlapping peptides that encompass the E6 and E7 oncoproteins of HPV type 16 (Best et al., 2012; Lin et al., 2010; Stern et al., 2012). Additionally, a phase II clinical trial of this peptide vaccine with the adjuvant Montanide ISA-51 resulted in the mounting of a complete vaccine-induced immunologic response (Best et al., 2012; Lin et al., 2010; Stern et al., 2012).

Protein vaccines are similar to peptide vaccines in many ways, but they can bypass MHC restriction since the protein contains a variety of antigenic epitopes (Lin et al., 2010; Ma et al., 2012). Additionally, protein vaccines are loaded onto MHC class II molecules, creating primarily a humoral response instead of a cell-mediated response (Lin et al., 2010; Ma et al., 2012). A phase II trial of the HspE7 protein-based vaccine, which is a chimeric protein composed of HPV-16 E7 and a Bacille Calmette-Guerin (BCG) heat shock protein (Hsp65), yielded modest results (S. Kumar et al., 2015; Ma et al., 2012). TA-CIN, a fusion protein composed of HPV-16 E6, E7, and L2, represents advancement in the field of HPV vaccination because it combines therapeutic as well as prophylactic vaccines. This protein-based vaccine has progressed to clinical trial (Lin et al., 2010; Stern et al., 2012).

The cell-based vaccine technique entails the pulsing of dendritic cells (DC) with an antigen (S. Kumar et al., 2015; Monie et al., 2009), allowing for the presentation of epitopes, such as those derived from HPV E7, in association with MHC molecules, and is capable of eliciting a high immunologic response (S. Kumar et al., 2015; Lin et al., 2010). A phase I study has shown the approach to be safe and immunogenic, and a phase II trial is underway (S. Kumar et al., 2015). However, the production of this vaccine is lengthy, taxing, and expensive (Lin et al., 2010; Monie et al., 2009) due to the need to isolate

immature dendritic cells from the patient, transfect or pulse the autologous DCs with the specific antigen, allow the DCs to mature, and expand the DCs *ex vivo* before injecting them back into the patient (Lin et al., 2010; Ma et al., 2012).

A live vector, consisting of either a bacteria or a virus, can be employed to deliver antigens such as those found in the E6 and E7 oncoproteins to the host APCs in order to enhance antigen presentation and the induction of a cell-mediated response (S. Kumar et al., 2015; Lin et al., 2010; Ma et al., 2012). These vectors generate a strong immune response by facilitating the spread and expansion of oncoproteins (S. Kumar et al., 2015; Lin et al., 2010; Ma et al., 2012). However, the disadvantage is that these live vectors could incite an immune response against the vector itself since it is intrinsically pathogenic and foreign to the host (S. Kumar et al., 2015). A bacterial vector-based vaccine composed of a genetically modified strain of *Listeria monocytogenes* fused to E7 has shown the ability to cause regression of solid tumors and has progressed to phase I clinical studies in oropharyngeal cancer patients (S. Kumar et al., 2015; Lin et al., 2010; Ma et al., 2012; Mirghani et al., 2015). Another group designed a vector vaccine using an integrase defective lentiviral vector (IDLV) to deliver a HPV-16 E7 protein fused to calreticulin (Elrefaey et al., 2014; Grasso et al., 2013; Lin et al., 2010). A preclinical study revealed that a single intramuscular injection eradicated 90% of early stage tumors (Elrefaey et al., 2014; Grasso et al., 2013). These encouraging outcomes along with emerging therapeutic vaccine trials may imply that an immunotherapeutic vaccine for immunocompetent patients shows a promising future (Elrefaey et al., 2014; Grasso et al., 2013).

10. Targeted Therapies Directed against Growth Factor Receptors

Current treatment for HNSCC patients is confined to standard therapies, such as irradiation, surgery, and chemotherapy (Fumagalli et al., 2015; Martinez-Useros & Garcia-Foncillas, 2015); and despite continued advances in these classic clinical modalities, survival rates remains comparable and many patients experience long-term side effects (Adams et al., 2014; Dorsey & Agulnik, 2013; Fumagalli et al., 2015; Inhestern et al., 2015). Consequently, advancements in molecular research have made the identification of targeted therapies an attractive therapeutic approach due to its purported reduced toxicity and improved efficacy (Adams et al., 2014; Dorsey & Agulnik, 2013).

We have come a long way in understanding the molecular biology of head and neck cancer over the past few decades (Purohit et al., 2013). Interestingly, the EGFR has been shown to be frequently elevated in over 90% of HNSCC patients (Burtness et al., 2014; Dorsey & Agulnik, 2013; Elrefaey et al., 2014; B. Kumar et al., 2007; Machiels et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015). EGFR contributes to the pathogenesis of HNSCC such that its overexpression is closely related to low survival, distant metastases, and radioresistance (Burtness et al., 2014; Dorsey & Agulnik, 2013; B. Kumar et al., 2007; Langer, 2012; Machiels et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015). Studies have indicated that low EGFR levels in HPV-positive tumors were correlated with favorable therapeutic outcomes, while high EGFR levels were associated with poor survival (Dorsey & Agulnik, 2013; Fumagalli et al., 2015; B. Kumar et al., 2007; B. Kumar et al., 2008; Mannarini et al., 2009).

The role of EGFR is to transmit signals to intracellular pathways that regulate a host of cellular activities including proliferation, cell cycle progression, apoptosis, migration, metastasis, differentiation and angiogenesis (Fumagalli et al., 2015; B. Kumar et al., 2008; Langer, 2012; Psyrri et al., 2013). Among the mechanisms attributed to overexpression of EGFR are deregulation of *TP53* and amplification of *EGFR* (Martinez-Useros & Garcia-Foncillas, 2015). Thus, this extracellular domain has been an attractive and prominent therapeutic target for treatment intervention (Fumagalli et al., 2015). Several agents directed against EGFR have been produced, of which monoclonal antibodies (mAb) and small tyrosine kinase inhibitors (TKIs) have been shown to be the most effective (Dorsey & Agulnik, 2013; Purohit et al., 2013). The mAbs bind to the extracellular binding domain of this receptor, while TKI's bind to the cytoplasmic side of EGFR and influence downstream molecular pathways (Elrefaey et al., 2014; Psyrri et al., 2013; Purohit et al., 2013; Zhang et al., 2014).

Cetuximab is a recombinant chimeric immunoglobulin (Ig)G mAb, specifically targeting the extracellular domain of EGFR (Dorsey & Agulnik, 2013; Elrefaey et al., 2014; Fumagalli et al., 2015; Psyrri et al., 2013). This mAb has been the most extensively studied of the anti-EGFR antibodies (Dorsey & Agulnik, 2013) and is the first and only targeted therapy approved for head and neck carcinoma (Bonilla-Velez et al., 2013; Burtness et al., 2014; Psyrri et al., 2013; Purohit et al., 2013; Sepiashvili et al., 2015). Food and Drug Administration (FDA) approval of cetuximab (Erbitux, Merck; Darmstadt, Germany) was established in 2006 after a phase III randomized study yielded remarkable results in the overall survival of HNSCC patients when cetuximab was used in conjunction with

radiotherapy (a survival of 45.6% *vs.* 36.4% for radiotherapy alone) (Adams et al., 2014; Bonilla-Velez et al., 2013; Bonner et al., 2006; Fumagalli et al., 2015; Machiels et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015; Psyrri et al., 2013). Therefore, cetuximab is recommended for the treatment of locally advanced HNSCC in combination with radiation and in recurrent/metastatic disease either as a monotherapy or in conjunction with platinum-based chemotherapy and 5-fluorouracil (Adams et al., 2014; Burtness et al., 2014; Dorsey & Agulnik, 2013; Martinez-Useros & Garcia-Foncillas, 2015; Psyrri et al., 2012; Psyrri et al., 2013). Several clinical trials are active including the Radiation Therapy Oncology Group (RTOG1016) trial, which compares cetuximab to cisplatin along with radiation in locally advanced disease (Adams et al., 2014; Blitzer et al., 2014; Bonilla-Velez et al., 2013; Langer, 2012; Laskar & Swain, 2015; Psyrri et al., 2012; Psyrri et al., 2013). This study will determine whether the less toxic cetuximab can replace cisplatin as part of a de-intensification protocol in HPV-derived HNSCC (Blitzer et al., 2014; Psyrri et al., 2013).

Other fully humanized IgG anti-EGFR antibodies under consideration include zalutumumab (HuMax-EGFr, Genmab, Copenhagen, Denmark) and panitumumab (Vectibix, Amgen; Thousand Oaks, CA, USA), and these are being investigated in phase II and III studies (Blitzer et al., 2014; Elrefaey et al., 2014; Martinez-Useros & Garcia-Foncillas, 2015; Psyrri et al., 2012; Psyrri et al., 2013). A phase II trial on nimotuzumab (YM Biosciences; Ontario, Canada), a recombinant humanized mAb, has demonstrated remarkable outcomes (Martinez-Useros & Garcia-Foncillas, 2015; Purohit et al., 2013).

These antibodies could potentially be used as substitutes for cetuximab (Elrefaey et al., 2014).

EGFR TKIs have also demonstrated some clinical activity in HNSCC but without as much success as seen with the mAbs (Psyrri et al., 2013; Zhang et al., 2014). The small molecule TKIs gefitinib and erlotinib showed no efficacy in recurring and metastasizing tumors (Psyrri et al., 2013; Purohit et al., 2013). A phase II trial of gefitinib on recurrent or metastatic head and neck cancer produced a low response rate (Psyrri et al., 2013), and ECOG-E1302, a phase III randomized study, evaluated gefitinib in addition to docetaxel in recurrent or metastatic head and neck cancer but was terminated before its completion (Psyrri et al., 2013). Despite these disappointments, other EGFR targets have yielded some early encouraging results (Urban et al., 2014). Lapatinib, a dual reversible tyrosine kinase inhibitor of EGFR/HER2, is in a phase III trial assessing its efficacy in the maintenance of treatment (Burtness et al., 2014; Fumagalli et al., 2015). Afatinib, also known as BIBW2992, is an irreversible dual tyrosine kinase inhibitor of EGFR/HER2 (Burtness et al., 2014; Psyrri et al., 2013). A randomized phase II trial is comparing cetuximab to afatinib in patients with recurrent or metastatic HNSCC where cisplatin has been unsuccessful (Burtness et al., 2014; Psyrri et al., 2013).

EGFR is involved in downstream intracellular pathways such as the PI3K/Akt/mTOR pathway. Alterations in the phosphoinositide 3-kinase (PI3K) pathway have been found in patients with head and neck cancers and appear even more predominately in patients with HPV-derived tumors (M. Lechner et al., 2013; Urban et al., 2014; Zhang et al., 2014). These alterations may contribute to tumor resistance to anti-EGFR therapy (Urban et al.,

2014). Hence, targeting PI3K is a reasonable strategy for OPSCC treatment, and trials in phases I and II are in progress (Urban et al., 2014). Research on the mammalian target of rapamycin (mTOR) inhibitors rapamycin, everolimus, and temsirolimus have shown mTOR suppression and delayed tumor advancement (Aderhold et al., 2015; Coppock et al., 2013; Dorsey & Agulnik, 2013). Additionally, rapamycin has been revealed to synergize with platinum-based chemotherapy in the eradication of OPSCC (Coppock et al., 2013). There are numerous trials in progress of mTOR inhibitors concomitant with different therapeutic modalities for head and neck carcinoma (Coppock et al., 2013).

Vascular endothelial growth factor (VEGF) is another type of growth factor and is considered one of the most critical angiogenic cytokines in tumor vasculogenesis (Dorsey & Agulnik, 2013; Hsu et al., 2014). Target agents have been developed to block its receptor, VEGFR. Bevacizumab is a monoclonal antibody against VEGFR that is being explored in conjunction to other anti-EGFR therapies (Argiris et al., 2013; Dorsey & Agulnik, 2013; Hsu et al., 2014). Sorafenib and sunitinib are tyrosine kinase inhibitors directed against VEGFR that have revealed notable therapeutic results in different human cancer cells with tolerable toxicity, and are showing encouraging results in OPSCC (Aderhold et al., 2013; Aderhold et al., 2015).

11. Targeted Therapies Directed against HPV Oncoproteins

Determining the molecular differences between HPV-dependent and HPV-independent head and neck cancers will be crucial in the discovery of therapeutic targets specific for HPV-dependent malignancies (Adams et al., 2014). Various investigations have indicated that the HPV oncogenes E6 and E7 or their substrates may be efficacious anti-

cancer targets (Griffin et al., 2006; Lui & Grandis, 2012). However, approaches targeting the oncogenes have only reached very early phases of development, in contrast to the late-phase developments attained by agents targeting growth factor receptors (Duenas-Gonzalez, Cetina, Coronel, & Cervantes-Madrid, 2012). Therapeutic agents targeting the viral oncoproteins include synthetic peptides (Zhao, Szekely, Bao, & Selivanova, 2010), RNA aptamers (Nicol et al., 2013; Stanley, 2012), ribozymes (Stanley, 2012; Zhao et al., 2010), transcription factors (Malecka et al., 2014), intrabodies (Malecka et al., 2014), anti-sense oligonucleotides (Malecka et al., 2014; Stanley, 2012), small interfering RNA (siRNA) (Caicedo-Granados et al., 2014; Stanley, 2012; Zhao et al., 2010), and small molecule inhibitors (Zhao et al., 2010). Because small molecule inhibitors can be easily delivered and absorbed by tumor cells (Zhao et al., 2010) and since they are flexible for medical use (Smukste, Bhalala, Persico, & Stockwell, 2006), they have gradually surfaced as a treatment option with notable efficacy and low toxicity (Figure 1) (Caicedo-Granados et al., 2014).

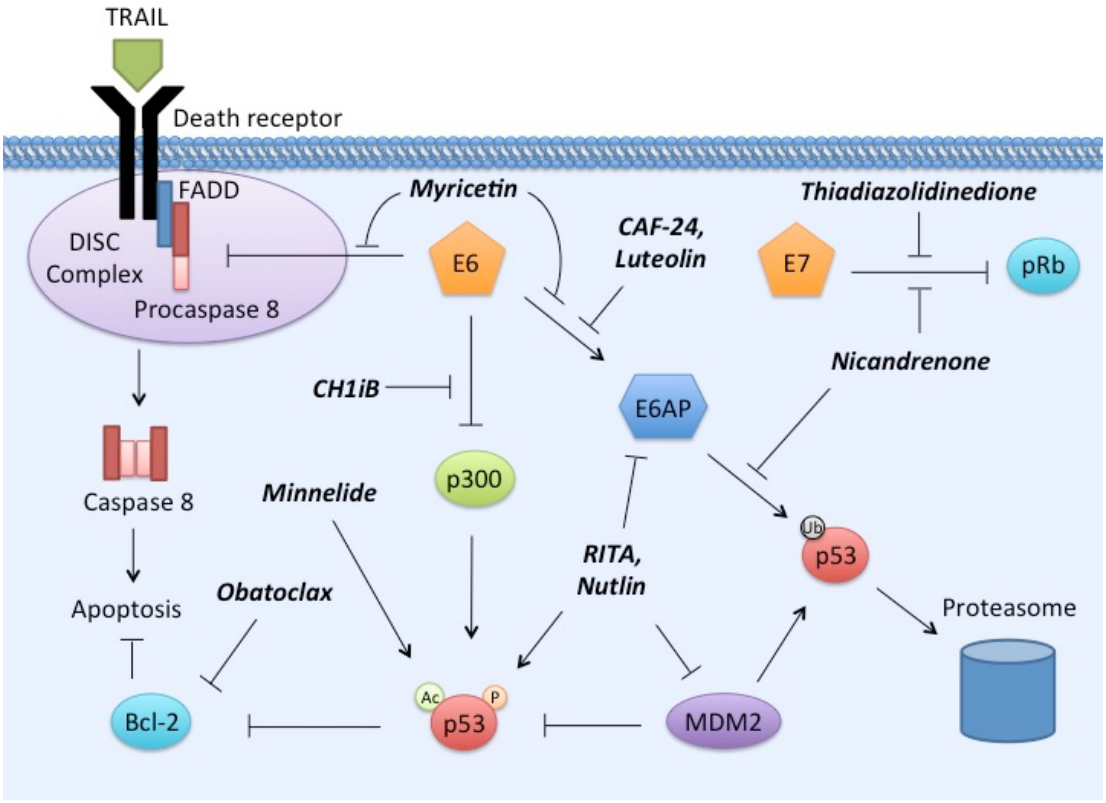

Figure 1. Involvement of small molecule inhibitors on cellular pathways affected by the E6 and E7 HPV oncoproteins.

The interaction between E6 and E6AP represents an attractive antiviral target, as agents that target this interaction may be able to inhibit the degradation of p53 and sensitize cells to agents that induce apoptosis (Caicedo-Granados et al., 2014; D'Abramo & Archambault, 2011; Stanley, 2012; Stern et al., 2012). One study has identified small molecules that bind to the oncoprotein E6 with great affinity (Cherry et al., 2013). In this study, the novel flavone CAF-24 and the naturally occurring flavonoid luteolin were shown to inhibit the E6-E6AP interaction by binding to the hydrophobic site between these two proteins (Cherry et al., 2013). This strategy inhibits the oncoproteins from binding to their cellular partners, thus inhibiting their oncogenic activities (Cherry et al., 2013; Malecka et al., 2014). Preventing the binding of E6AP and thus the degradation of p53 can reactivate

the apoptotic pathways, enhancing the outcome of available therapies (Caicedo-Granados et al., 2014; Cherry et al., 2013; Cho et al., 2000).

A small molecule that has been widely studied in multiple types of cancer is the p53 protector, RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) (Issaeva et al., 2004; Zhao et al., 2010). This molecule targets p53 by changing its conformation and protecting it from binding to molecules such as E6AP and E6 that facilitate ubiquitination (D'Abramo & Archambault, 2011; Stanley, 2012; Zhao et al., 2010). In this way, p53 is rescued and the apoptotic pathway reactivated, leading to the loss of tumor cells (Zhao et al., 2010).

A similar approach is taken by the non-peptide small molecule compound Nutlin-3A, an imidazoline analog and potent MDM2 antagonist. Nutlin causes substantial cell death in a variety of wild-type p53 expressing cell lines (Caicedo-Granados et al., 2014); however, its activity appears to be moderate as compared to RITA (Zhao et al., 2010). Another promising molecule that reactivates the wild-type p53 is Minnelide, a triptolide analog, which has shown to induce apoptosis in HPV-positive HNSCC tumors *in vitro* as well as *in vivo* (Caicedo-Granados et al., 2014). CH1iB is a novel small molecule that also reactivates p53 function by inhibiting E6 from binding to p300 and thereby allowing p300 to acetylate p53 (Xie et al., 2014). This acetylation increases p53 stability and transcriptional activity, prompting the active p53 tumor suppressor pathway to induce apoptosis when cells are treated with chemotherapeutic agents (Xie et al., 2014). A preclinical study of Obatoclax, a small molecule antagonist of the Bcl-2 family (B-cell lymphoma 2 (Bcl-2) is a downstream substrate of E6 that is associated with resistance to

treatment (B. Kumar et al., 2008)), indicates some therapeutic value in the treatment of oropharyngeal carcinoma (Yazbeck, Li, Grandis, Zang, & Johnson, 2014).

Another attractive target is the interaction of E6 and caspase 8, a protein involved in the extrinsic apoptotic pathway (Yuan, Filippova, Tungteakkhun, Duerksen-Hughes, & Krstenansky, 2012). The extrinsic apoptotic pathway can be activated by several TNF-family ligands including TNF-related apoptosis-inducing ligand (TRAIL). TRAIL can initiate apoptosis in tumor cells with expression of TRAIL-specific receptors, namely DR4 and DR5 (Garnett, Filippova, & Duerksen-Hughes, 2007), and TRAIL-therapy is considered a promising anti-tumor approach. Binding of ligands to the receptor activates the apoptotic cascade, which starts with the formation of the death-inducing signaling complex (DISC) complex composed, in many instances, of the receptor, FADD and the initiator caspase, procaspase 8. The assemblage of this complex results in cleavage and activation of procaspase 8. E6 interferes with this process by binding to procaspase 8 and FADD, accelerating their degradation and preventing the successful completion of the apoptotic cascade (Filippova et al., 2007; Filippova et al., 2004; Tungteakkhun et al., 2010; Tungteakkhun et al., 2008). If therapeutic agents such as small molecules could inhibit E6 from binding to procaspase 8 and FADD, it would restore the normal functioning of the apoptosis pathway. Proof of principle for this approach was demonstrated by the flavonol myricetin, which was able to prevent the binding of E6 to caspase 8, showing potential for reactivating the extrinsic apoptotic pathway (Yuan, Filippova, Tungteakkhun, et al., 2012). Further studies on the identification, optimization and evaluation of small molecules of E6 inhibitors are currently underway.

Another strategy is to inhibit the interaction between E7 and pRb, thereby preventing E7 from inhibiting pRB's ability to inhibit cell division. The small compound thiadiazolidinedione inhibits HPV-E7 from disrupting the pRb-E2F complex by blocking the E7-pRb interaction (Fera et al., 2012). Lastly, a small compound, namely nicandrenone, has demonstrated the ability to target the sites of both the E6-p53 and E7-pRb1 interactions, thereby blocking the transformative activities of both viral oncoproteins (Shaikh, Sanehi, & Rawal, 2012). All the above strategies can lead to the development of efficient therapies against HPV-driven OPSCC and could be used in combination with current therapies to induce tumor cell death and reduce the undesirable side effects of current treatments.

Research into small molecules useful for the treatment of HPV-dependent cancers is ongoing and encouraging. However, concepts developed during studies conducted on cervical cancer will have to be assimilated and translated to oropharyngeal carcinoma. Further developments in our understanding of the molecular biology underlying the development of HNSCC will be necessary to refine the efficacy of these early phase agents.

12. Conclusions and Future Directions

The current epidemic of HNSCC has sparked significant interest in the role of HPV in oncogenesis, and the emergence of HPV-positive head and neck cancer has shifted the demographic of HNSCC from an older population to a younger generation. Current treatments, which consist of transoral surgery, platinum-based chemotherapy, and intensity-modulated radiotherapy, are increasingly recognized as requiring

improvements. While advances in standard therapies have improved outcomes, the new group of younger patients is at high risk of morbidity and consequently a compromised quality of life. Therefore, the demand for major progress in the therapy and diagnosis of HPV-associated carcinoma remains current and compelling (Haedicke & Iftner, 2013). The better prognosis of HPV-related OPSCC has broached topics of de-escalation strategies (Hinni et al., 2015), leading to the emergence of various de-intensification trials for HNSCC. With this concern in mind, standardizing a screening method for HPV status would help in diagnosing and delivering appropriate treatments to this subpopulation. The commercially available HPV prophylactic vaccines have had a profound effect in the prevention of HPV infection in the context of cervical cancer, but their efficacy has not yet been proven in the context of HPV-dependent head and neck carcinomas. Ongoing trials are anticipated to address this issue. A preventive vaccine would mitigate the epidemic long-term, but will not address the more urgent issue of treating patients with existing HPV infections. Hence, the development of therapeutic vaccines has the potential to meet a pressing need for better treatments of HPV-associated tumors in immunocompetent OPSCC patients. Additionally, targeted therapies of growth factors potentially have a more widespread use, and they have progressed in clinical trials, though with mixed results and varying success.

Several advances in biotherapy have led to the identification of a number of small molecular compounds with the potential for contributing to the development of less toxic treatments. The field of small molecular targeted therapy is in its infancy, but current findings are encouraging, advocating for the rapid progression of the field. The studies

presented above reveal the urgency of the burden and the impetus to identify better targets and antiviral therapies effective in attenuating the incidence of HPV infection and counteracting the growing epidemic of HPV-associated head and neck cancers (D'Abramo & Archambault, 2011).

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Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER TWO

HPV^{+} HNSCC XENOGRAFT MODEL DEVELOPMENT AND *IN VIVO* ANTITUMOR EFFICACY STUDY OF SPINACINE

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1. Introduction

High-risk types of human papillomaviruses (HPV) are responsible for 99% of cervical cancer cases, and 70% of head and neck (HN) cancer, as well as 65% of vaginal, penile, and vulvar cancer (Cavallo, 2021; Szymonowicz & Chen, 2020). While the prevalence of cervical cancer has gradually declined in the US in the last 30 years due to Pap smear screening and preventive measures, the incidence of HPV^+ HN cancers has steadily increased within the same period (Adams et al., 2014; Sabatini & Chiocca, 2020). The frequency of HPV^+ head and neck squamous cell carcinoma (HNSCC) have dramatically increased more than two-fold in the last decades, affecting mostly young white males (Chaturvedi et al., 2013; Gillison et al., 2012; Gillison et al., 2014; McDonald, Qendri, Berkhof, de Melker, & Bogaards, 2017; Sabatini & Chiocca, 2020). These numbers underscore the need to develop more targeted and antiviral treatments against HPV-related cancers.

With rising incidences in HNSCC, many efforts and measures to prevent and treat HPV cancers have been increasing. One noteworthy evidence of progress is the recent approval of Gardasil 9 in the prevention of oropharyngeal and head and neck carcinoma (Diana & Corica, 2021). The prophylactic HPV vaccine can now prevent the development of cervical, vulvar, vaginal, anal, and oropharyngeal cancer by targeting high-risk HPV types 16, 18, 31, 33, 45, 52, 58, as well as genital warts caused by types low-risk types 6, and 11. Vaccines rely on a functional immune system to elicit protection, and as great of a stride as they are in the prevention of HPV illnesses, they are not effective interventions

for individuals already infected with HPV or for those suffering from immunodeficiencies (Reusser, Downing, Guidry, & Tyring, 2015).

In terms of current therapeutic options, surgical treatment is often employed to cure cervical cancer in the early stages, but it is not always easy to use this approach in HN cancers or in laryngeal papillomatosis, due to the need to maintain the normal structure of the airway and to avoid pulmonary spread. Following surgical excision, however, HPV-associated cancers frequently return (Rein & Kurbacher, 2001), especially when diagnosed at later stages and/or when present in immunocompromised individuals (Reusser et al., 2015). Chemo- and radiotherapy are commonly administered in combination or following a relapse and are based on the idea that they can damage DNA, which will trigger apoptotic death of the cancer cells. Unfortunately, these treatments also damage the DNA of normal cells, particularly cells that divide rapidly such as those within the bone marrow, resulting in a weakening of the immune system. Hence, it may be difficult to pair these classical approaches even with some therapeutic vaccines currently under development. Clearly, there remains a compelling and urgent need to develop more targeted and better treatment options for patients with head and neck cancer and other HPV-associated malignancies.

Chemo- and radiotherapies that rely on the induction of apoptosis in tumor cells are relatively ineffective largely due to the HPV oncogene E6, which increases resistance by disrupting both the intrinsic and extrinsic apoptotic pathways **(Figure 2).** The first-described and best-known target of HPV E6 is the tumor suppressor p53, an important mediator of intrinsic apoptotic pathways.

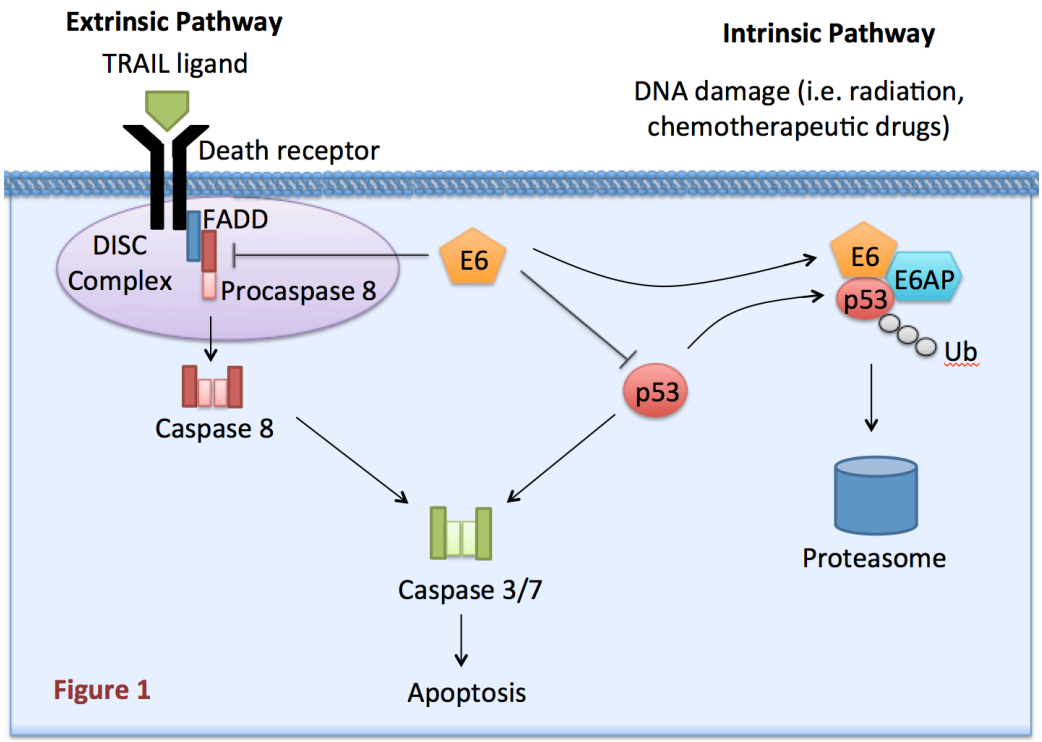


Figure 2 – Schematic of E6 targets in cellular apoptotic pathways.

E6 proteins from high-risk HPV bind to E6AP, an E3 ubiquitin ligase. Then, the E6/E6AP complex binds to the core domain of p53, and E6AP catalyzes the transfer of ubiquitin to p53. The accelerated ubiquitination leads to its rapid proteasomal degradation, thereby increasing the survival and growth of the transformed cells (Huibregtse, Scheffner, & Howley, 1991; Scheffner, Huibregtse, Vierstra, & Howley, 1993; Scheffner et al., 1990). Our laboratory has discovered that HPV16 E6 also inhibits activation of extrinsic apoptotic pathways, such as those triggered by tumor necrosis factor (TNF), Fas ligand and TRAIL. E6 subverts this pathway by binding to and inactivating several molecules involved in these signaling complexes including caspase 8 (Filippova et al., 2007), FADD (Filippova et al., 2004), and TNF R1/TRAIL (Filippova, Song, Connolly, Dermody, & Duerksen-Hughes, 2002). E6 can bind to the death effector domains (DEDs) of FADD and procaspase-8 and

accelerate their degradation (Filippova et al., 2009; Filippova et al., 2004; Garnett, Filippova, & Duerksen-Hughes, 2006; Tungteakkhun et al., 2010). The resulting lower amounts of FADD and procaspase 8 in E6-expressing cells then hinders formation of the apoptotic Death Inducing Signaling Complex (DISC) that would normally be triggered by members of the TNF superfamily, thereby compromising the ability of TNF, FasL and TRAIL to initiate apoptosis (Filippova, Brown-Bryan, Casiano, & Duerksen-Hughes, 2005; Filippova et al., 2004; Filippova et al., 2002; Garnett et al., 2006). As a result, engagement of either the extrinsic or the intrinsic apoptotic pathways fails to result in the transduction of the intended death signal because the mediator molecules – p53 in the case of the intrinsic pathway, and caspase 8 and FADD in the case of the extrinsic pathway – are missing.

Hence, in cases where therapies function by activating apoptosis, including those based on TRAIL and cisplatin, they can become handicapped in their ability to effectively treat HPV-associated malignancies. Our laboratory's work over the past couple of decades, along with that of other labs, has implicated E6 as an excellent potential antiviral target for therapeutic intervention since E6 specifically obstructs the apoptotic pathways (Yuan, Filippova, Krstenansky, & Duerksen-Hughes, 2016). Moreover, although *E6* and *E7* are both considered indispensable for transformation efficiency, *E6* can, in some instances, immortalize cells in the absence of *E7*, indicating that *E6*-dependent mechanisms underlying HPV-associated carcinogenesis are of vital importance (Band, De Caprio, Delmolino, Kulesa, & Sager, 1991; Wazer, Liu, Chu, Gao, & Band, 1995). Therefore, an approach based on inhibiting the ability of E6 to mediate the rapid degradation of its

cellular partners has the potential to re-sensitize HPV$^+$ cells to inducers of apoptosis and could therefore make certain existing cancer treatments more effective for those suffering from HPV-associated malignancies.

Since HPV-associated malignancies remain a significant clinical challenge, our approach~~premise~~ to address this shortcoming is by identifying small molecules that can block E6 binding to its cellular partners so as to re-establish the ability of HPV$^+$ cells to respond to apoptotic signals. Small molecules possess numerous advantages over peptides or other biological molecules because they are more stable, penetrate target cells more easily, and can more readily be modified and optimized by organic chemists during drug development. Moreover, small molecules have already been used to inhibit important signal transduction pathways involved in breast, colon, pancreatic and lung cancer formation (Yi, Maksimoska, Marmorstein, & Kissil, 2010). Some progress has been made in identifying small molecules that can interfere with E6 activities (Baleja et al., 2006; Beerheide et al., 1999; Beerheide, Sim, Tan, Bernard, & Ting, 2000; Cherry et al., 2013; Choulier et al., 2013; Malecka et al., 2014), but no studies have yet combined E6 inhibitors with apoptosis-inducing agents.

Human TNF-related apoptosis inducing ligand (TRAIL) is a cytokine that induces cell death through the extrinsic apoptotic pathway. The C-terminal part of TRAIL is responsible for induction of apoptosis through binding to TRAIL receptors, while the N-terminal encodes the transmembrane domain. TRAIL-based therapies have elicited significant interest mainly due to the ability to kill cancer cells while sparing most noncancerous cells (Ashkenazi et al., 1999; Garnett et al., 2006; Wiley et al., 1995). TRAIL

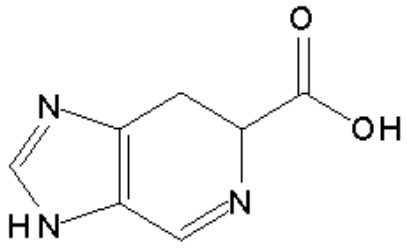
receptors 1 and 2 (TRAIL R1 and TRAIL R2, also known as DR4 and DR5, respectively) are highly expressed on a large number of solid and hematologic cancers, making these tumors sensitive to apoptosis induced both by TRAIL itself and by antibodies to the receptor (Bellail, Qi, Mulligan, Chhabra, & Hao, 2009; Buchsbaum et al., 2003; Chuntharapai et al., 2001; Ichikawa et al., 2001; Tanaka, Sugimachi, Shirabe, Shimada, & Wands, 2000; Younes & Kadin, 2003). In addition to the anticancer properties, a growing body of evidence suggests that TRAIL may play an important role in host defense against viral infection. Notably, TRAIL is expressed on a variety of cells involved in host defense against viral infection including cytotoxic T cells (Jeremias, Herr, Boehler, & Debatin, 1998; Kayagaki et al., 1999), dendritic cells (Fanger, Maliszewski, Schooley, & Griffith, 1999), monocytes/macrophages (Griffith et al., 1999), and natural killer (NK) cells (Sato et al., 2001; Zamai et al., 1998). TRAIL-induced apoptosis is initiated by the binding of TRAIL to death receptor (DR4) (Pan, O'Rourke, et al., 1997), and/or death receptor 5 (DR5) (Pan, Ni, et al., 1997; Sheridan et al., 1997). TRAIL forms a homotrimer and associates with three receptor monomers (Mongkolsapaya et al., 1999). Homotypic, protein-protein interactions between conserved death domains and death effector domains lead to the assembly of Fas-associated death domain (FADD) and procaspase(s) 8/10 molecules at the C-terminus of the receptors to form the death inducing signaling complex (DISC) (Kischkel et al., 2000; Sprick et al., 2000). Activation of caspase(s) 8/10 at the DISC is then followed by the activation of executioner caspases, such as caspases 3 and 7, the cleavage of cellular substrates, and the loss of cell viability. However, TRAIL therapy has a major

limitation as a large number of cancers develop resistance toward TRAIL (de Miguel, Lemke, Anel, Walczak, & Martinez-Lostao, 2016) (Trivedi & Mishra, 2015).

Elucidation of the molecular targets and signaling pathways responsible for TRAIL resistance demonstrated that this could be due to aberrant protein synthesis, protein misfolding, ubiquitin regulated death receptor expression, metabolic pathways, epigenetic deregulation, and metastasis (Trivedi & Mishra, 2015). Discovery of novel drugs that could inhibit the defective cellular processes may restore TRAIL sensitivity and re-sensitize TRAIL resistant cancer cells toward TRAIL-induced apoptosis (De Miguel, Gallego-Lleyda, Ayuso, Erviti-Ardanaz, et al., 2016; De Miguel, Gallego-Lleyda, Ayuso, Pawlak, et al., 2016; de Miguel, Lemke, et al., 2016). In the case of HPV-mediated malignancies, the only carcinogenic agent inducing the transformation of the cells is high risk HPV and, hence, the expression of the viral oncogene E6. The role of E6 in TRAIL-mediated apoptosis is to inhibit the binding of FADD (Filippova et al., 2004) and caspase 8 (Filippova et al., 2007), and thus deactivate the infected cells from undergoing apoptosis through the extrinsic pathway. Therefore, eliminating the oncogenic activities of HPV E6 is predicted to restore the sensitivity of host cells to apoptotic signals such as those delivered by TRAIL.

We developed a bead-based assay for high-throughput screening (HTS), based on PerkinElmer's AlphaScreen® technology, and used it to query a 2,000-compound diverse library (Actiprobe-2K) obtained from TimTec, for small molecules capable of inhibiting E6/caspase 8 binding. Hit identification, lead identification and its optimization were evaluated in a multi-step process including primary library screening, dose-response analysis, counter-screening, optimization based on SAR analysis, and finally, an isothermal

scanning calorimetric (ITC) assay for kinetic measurements. At the conclusion of this process, we had identified one candidate from the library queried: the imidazole amino acid derivative of histidine, spinacine **(Figure 3)** (Yuan et al., 2016; Yuan, Filippova, Tungteakkhun, et al., 2012). Spinacine is a benzimidazole, a derivative of the amino acid histidine, and a natural product from food sources such as dairy products, spinach and the roots of Panax ginseng (Pellegrino & Resmini, 1996; Restani, Campagner, Fiecchi, Resmini, & Galli, 1988). In addition, it can be isolated from crabs and shark liver. There is no available information on the biological activity of spinacine with the exception of one set of toxicological data in rats showing no toxic effect from an oral dose of 300 mg/kg body weight per day for 13 weeks (Galli et al., 1989).



Spinacine

Figure 3 – Structure of Spinacine

Earlier work in our lab had demonstrated that spinacine, unlike other potential E6-inhibitory molecules described by others, inhibits both E6/caspase 8 and E6/E6AP interactions. This unique property allowed us to simultaneously restore both the intrinsic and extrinsic apoptotic pathways, potentially amplifying treatment effectiveness. Importantly, we found that spinacine, selected for its ability to block E6/caspase 8 binding, sensitizes E6-expressing cells to death mediated by both TRAIL and chemotherapeutic agents such as cisplatin (Yuan et al., 2016; Yuan, Filippova, Tungteakkhun, et al., 2012). As predicted, these small molecules restored cellular levels of p53 and procaspase 8 (Yuan et al., 2016), thus validating our working model. Our hypothesis was that using spinacine as an inhibitor of E6 will restore the apoptotic pathways and reduce or eliminate HPV^+ tumor growth when combined with apoptosis-induced therapy. Hence, we wanted to test the ability of our lead compound, spinacine, in reducing or eliminating the growth of tumors in a xenograft murine model. This study directly compared the effectiveness of spinacine in partnering with TRAIL and with cisplatin. Success in this project would lay the groundwork for future clinical trials of spinacine as well as its chemical optimization.

2. Results

In preparation for assessing the use of spinacine in further clinical trials, we established an optimized HPV^+ tumor xenograft model to determine spinacine's antitumor efficacy in the context of this model. First, we extended our previously established HPV^+ tumor xenograft model to include not only cervical cancer but also head and neck cancer xenografts. Moreover, we improved our *in vivo* tumor models to produce more consistent

tumor growth and implemented a state-of-the-art tumor visualization technology. Once spinacine's toxicity level was assessed and its dose optimized, and sufficient hrTRAIL was purified and collected for the tumor inhibition study, we were able to evaluate the antitumor effects of spinacine in synergy with hrTRAIL as well as with the chemotherapeutic drug cisplatin in our established HPV^+ HN tumor *in vivo* model.

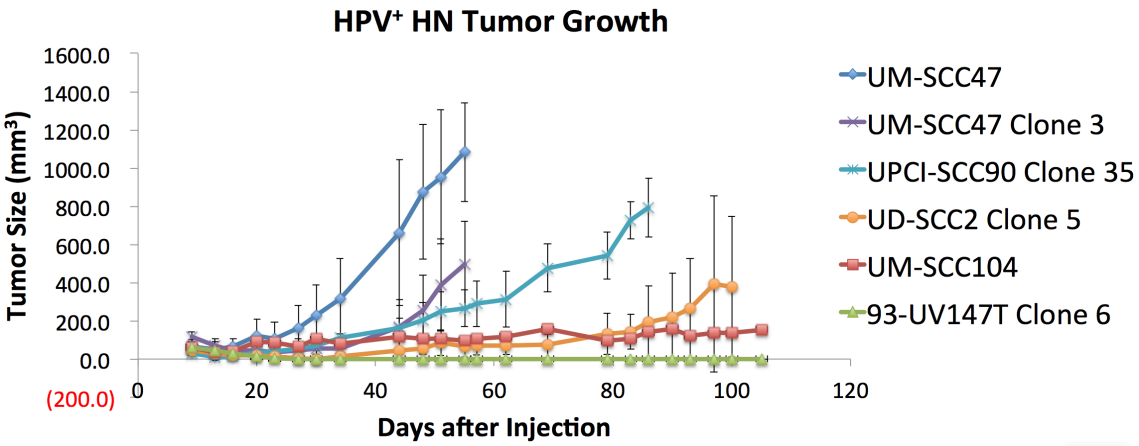
2.1 Development of a system that can effectively test small molecules in vivo.

We focused on extending and enhancing our existing HPV^+ tumor xenograft model. First, we expanded our cancer xenograft model to incorporate an HPV^+ head and neck cancer model in addition to our cervical cancer model. Then we enhanced the model to obtain better and more consistent tumor growth by using Matrigel, a basement membrane matrix. Additionally, we applied luciferase technology to enable state-of-the-art tumor visualization in a real-time manner in live animals.

2.1a) HN cancer as an HPV^+ tumor xenograft model. Our laboratory has previously performed studies using a mouse xenograft model injected with the HPV^+ SiHa cervical cancer cells (Filippova et al., 2014). In order to assess the effectiveness of our compounds in the context of HN tumors, we proceeded to develop an HPV^+ HN cancer model by comparing tumor growths of six HPV^+ HN cell lines (UM-SCC47, UM-SCC104, 93UV-147-Up-Clone 6, UM-SCC47-TC-Clone 3, UPCI-SCC90-Up-Clone 35, and UD-SCC-2TC-Clone 5) in nude mice. The growth curve clearly demonstrated that of all the cell lines tested, UM-SCC47 grew the fastest **(Figure 4)**. The HPV^+ HN cell line UM-SCC47 was chosen not only

because they develop fast-forming tumors, but also because they displayed significant sensitization to TRAIL following pretreatment with spinacine (Yuan 2012). Hence, these cells were selected for the creation of a HN xenograft model for the *in vivo* studies described below. In addition, we note that these cells have previously been successfully used in a similar xenograft model (Caicedo-Granados et al., 2014). In conclusion, in the experiments described in section 2, we will employ a xenograft model where UM-SCC47 cells model HPV^+ HN cancer.

A)



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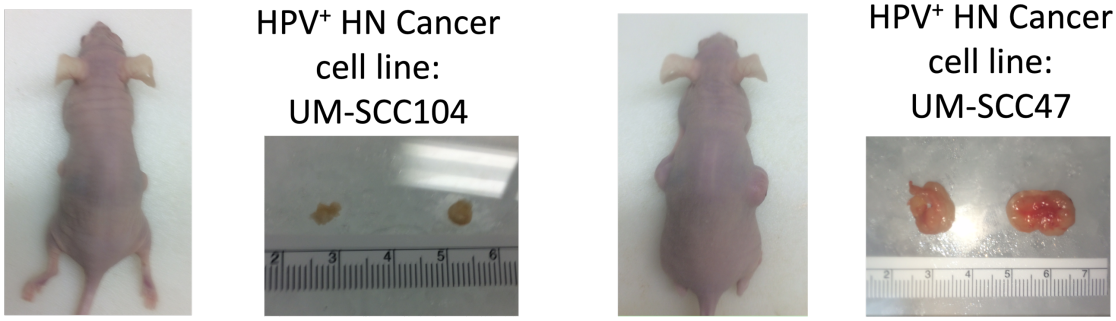


Figure 4 – HPV$^+$ head and neck cancer cell lines tested in the xenograft model. **(A)** Six different HPV$^+$ head and neck cancer cells were injected into nude mice and growth was monitored over time. Each cell line was tested on two mice with 7.5x10^6 cells injected into each flank. From all the HPV$^+$ HN cell lines, UM-SCC47 was shown to be the fastest growing. **(B)** Representative pictures of the slowest and fastest growing tumors from the HPV$^+$ HN cell lines engrafted.

2.1b) Matrigel incorporation into the HPV$^+$ tumor xenograft model. Optimization of *in vivo* tumor models can enable more consistent tumor growth. Matrigel, an extracellular basement membrane gel that aids in the implantation and growth of tumors *in vivo*, allows tumors to remain in a confined area in mice. Our protocol tested two HPV$^+$ HN cancer cells (UM-SCC47 and UPCI-SCC90) and split the groups into two: one with Matrigel and the other without Matrigel. We were able to demonstrate that tumors grow at a greater speed and at a more consistent rate when Matrigel was added **(Figure 5A-B)**. These study results indicate that Matrigel enhances the progression of our tumor growth; therefore, we implemented Matrigel usage into our subsequent tumor inhibition studies.

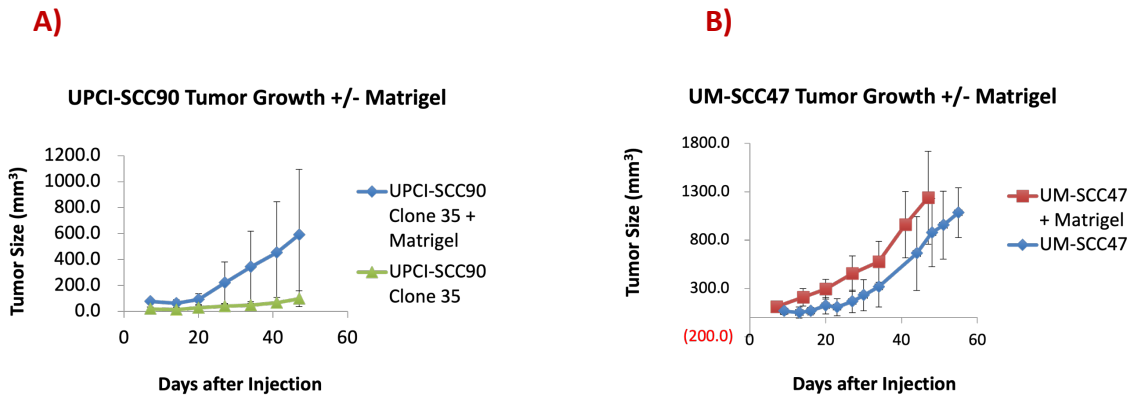
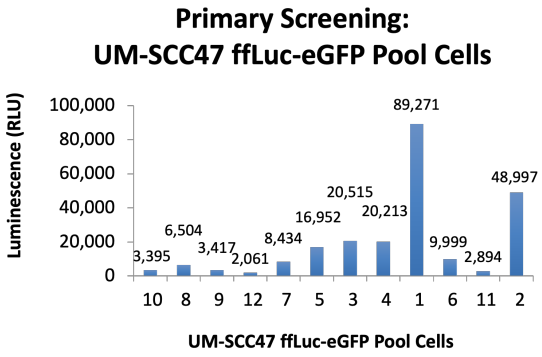


Figure 5 – (A) 1.5x10^7 of UPCI-SCC90 Clone 35 cells or **(B)** 1x10^7 of UM-SCC47 cells were injected into each flank and were grown over time. 100 µL of Matrigel were added to the cancer cells in one group, whereas the other group did not contain any Matrigel. In conclusion, Matrigel assisted in the rapid and consistent growth of the tumor.

2.1c) Luciferase technology and bioluminescence imaging in the xenograft model. Bioluminescence imaging has been widely used in animal experiments, and luciferase technology offers the ability to detect tumor growth and metastasis visualization in live rodents without the need for surgical and/or terminal procedures. To utilize this state-of-the-art technology, we utilized tumor cells expressing high levels of *firefly luciferase*, utilizing lentiviral transduction to create HPV^{+} head and neck UM-SCC47 *ffLuc* cells. Based on the primary screening, we selected pools of cells expressing luciferase at a high level and labeled them Pools 1 through 4 **(Figure 6a)**. A secondary screening confirmed that UM-SCC47 Pool 1 cells had the highest bioluminescence intensity (BLI) **(Figure 6b)** and was selected for tumor injection. When tumors became detectable, mice were imaged using IVIS Lumina III (PerkinElmer). This study successfully detected *firefly luciferase* in UM-SCC47 *ffLuc* cells **(Figure 6d).** This technology enabled us to visualize and assess tumor growth more accurately in-real-time through bioluminescence visualization.

A)



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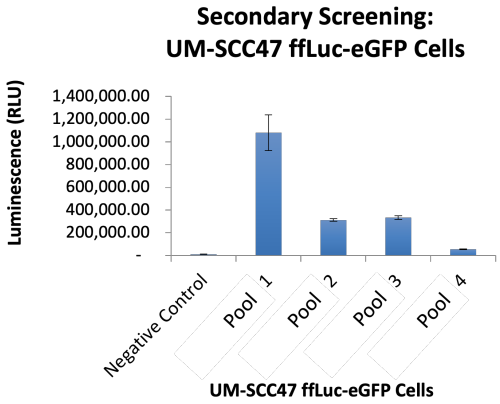

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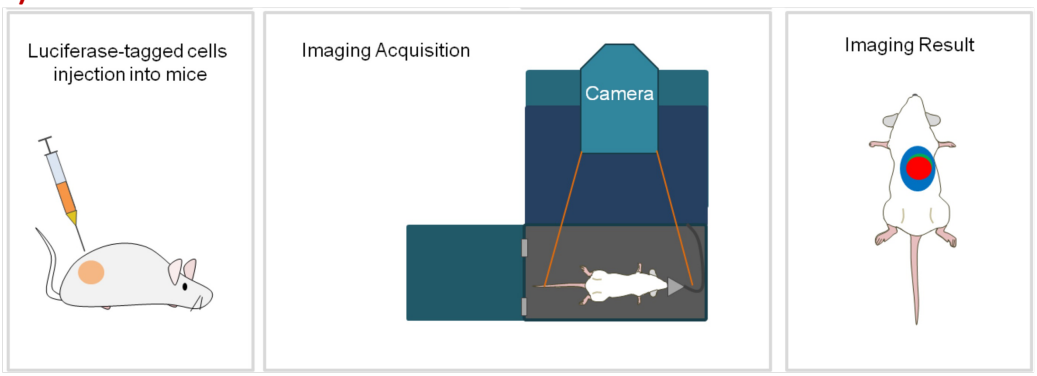

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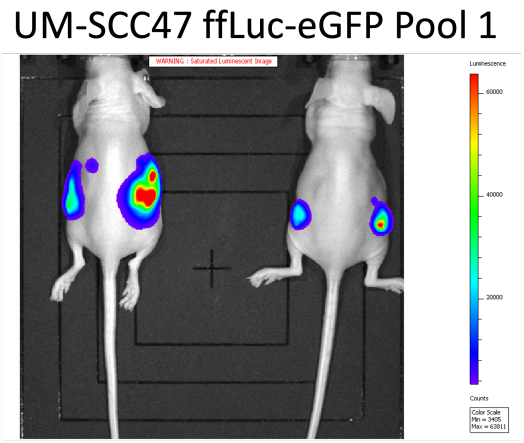


Figure 6 – Relative bioluminescence reading of UM-SCC47 *ffLuc* pool cells from **(A)** primary and **(B)** secondary screenings. **(C)** Procedure to acquire images of luminescence expressing tumors in a mouse xenograft model. **(D)** Visualization of the HPV^{+} head and neck cancer model UM-SCC47 *ffLuc*-eGFP Pool 1.

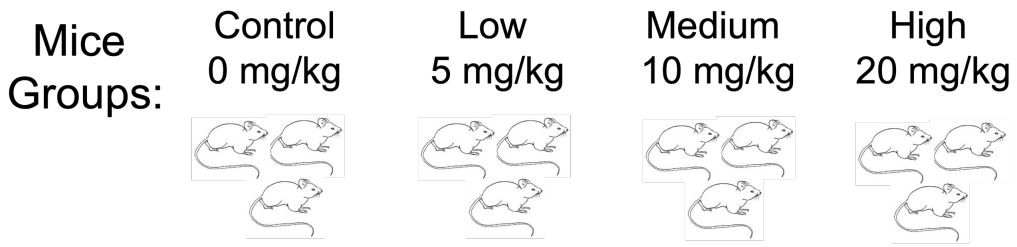
2.2 Tumor Growth Inhibition Study in an HPV$^+$ xenograft model.

Before testing the efficacy of spinacine, we purified, collected, and tested hrTRAIL in cells before using the material in mice in the tumor inhibition study. We also tested the toxicity levels of spinacine by performing a dose-finding experiment. After an optimal dose of treatment in mice was determined, we assessed the ability of spinacine to synergize with hrTRAIL and/or the DNA damaging drug cisplatin to inhibit tumor growth in the *in vivo* models. We used the murine xenograft models established in the previous section and determined the extent to which each of the treatment plans and combinations reduced or eliminated tumor growth.

2.2a) Spinacine dose optimization. We performed a dose-response study in preparation for the antitumor efficacy experiment. We tested different concentrations of the spinacine in CD1 mice (Crl:CD-1-022), and we selected our initial *in vivo* doses of the small molecule to be either low, medium, high dose or vehicle as shown in **Figure 7A**. Four groups of 3 mice each were injected intraperitoneally with the indicated concentrations of spinacine on days 1 and 2. After a break on the third day, this cycle was repeated 5 times for a total treatment period of 2 ½ weeks (**Figure 7B)**. Animals were weighed every 2-3 days and observed for changes indicative of declines in health, such as level of activity, ocular porphyrin, hunched posture, drastic weight loss, lack of fur grooming **(Figure 7C-D)**. None of these clinical signs of decline in health or mortality were observed. Moreover, these observations were consistent with hematological results as well as post-mortem evaluation of major internal organs in the control and high dose groups **(Figure 7E)**, none

of which showed signs of toxicity. This data indicates that spinacine is not toxic to mice at doses up to and including 20 mg/kg.

A)



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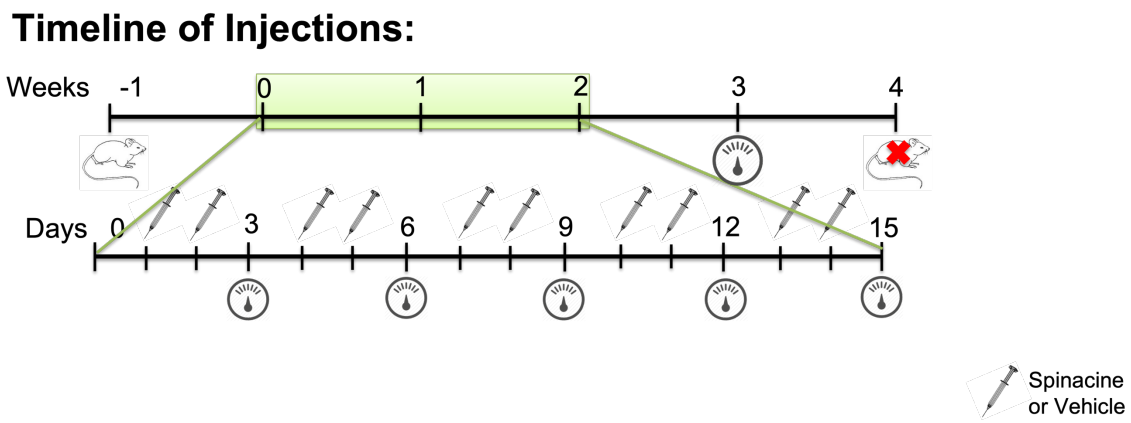

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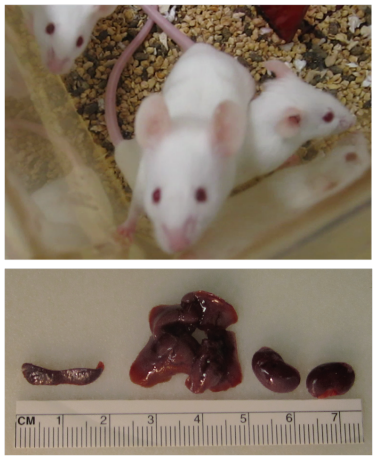

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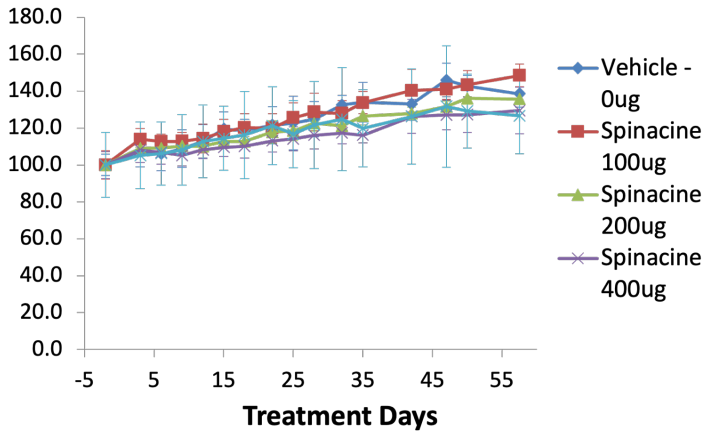


E)

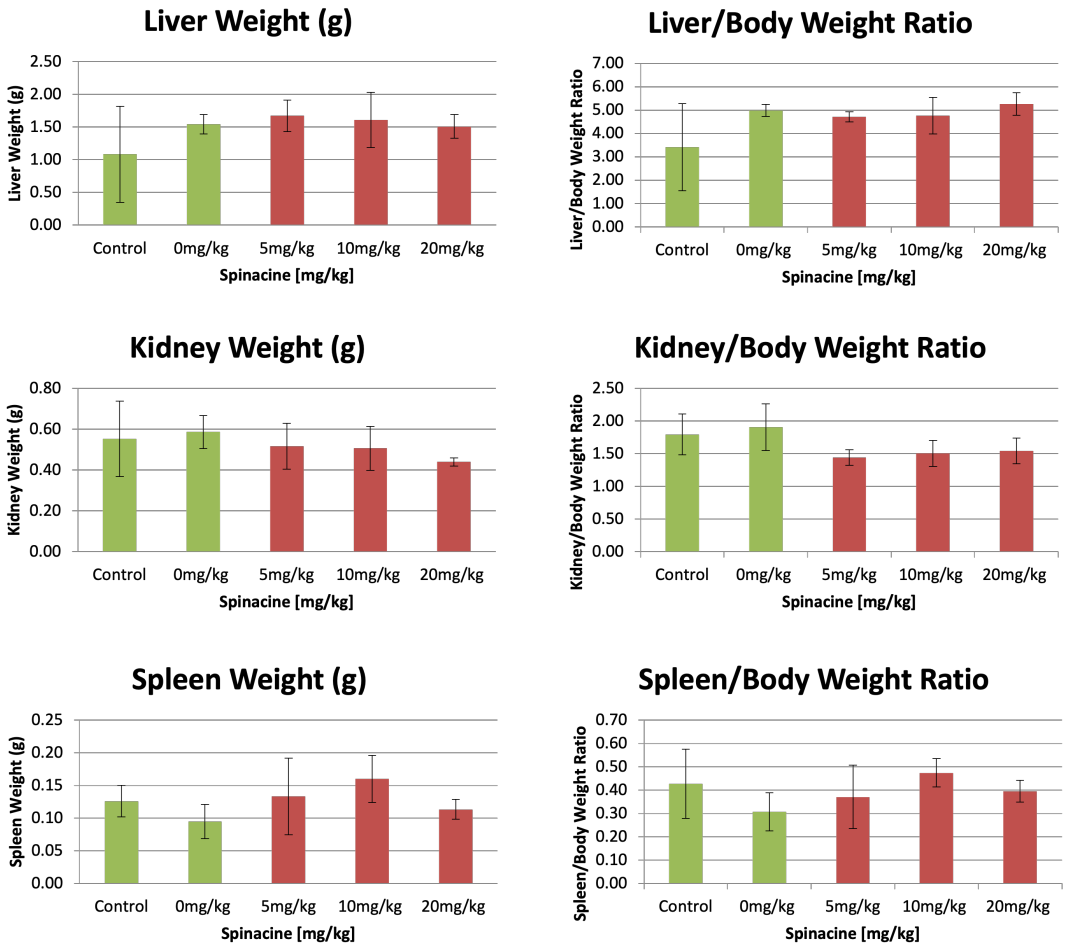


Figure 7 – Spinacine Toxicity Study. **(A)** Group of treatments. **(B)** Scheme of treatment timeline. **(C)** Representative pictures demonstrating mouse physical health (top) and showing the internal organs (bottom). Pictures and organs are from the group treated with the highest concentration of spinacine. **(D)** Average mouse weight for each group is depicted for the duration of the experiment. **(E)** Evaluation of post-mortem major organs by group average weights.

2.2b) hrTRAIL Protein Purification. Because TRAIL-based therapies can kill tumor cells through the extrinsic apoptotic pathway, while sparing most normal cells, we chose to begin by testing the effect of a spinacine and human recombinant TRAIL (hrTRAIL) combination on HPV^{+} tumor growth. To obtain the amount of hrTRAIL necessary for the antitumor efficacy animal experiment (25 mg), we cloned the part of the protein containing the site that binds to the TRAIL receptors into the *E. coli* protein expression vector *pTriEx-4,* produced the protein **(Table 1)**, and demonstrated its apoptotic activity **(Figure 8)**.

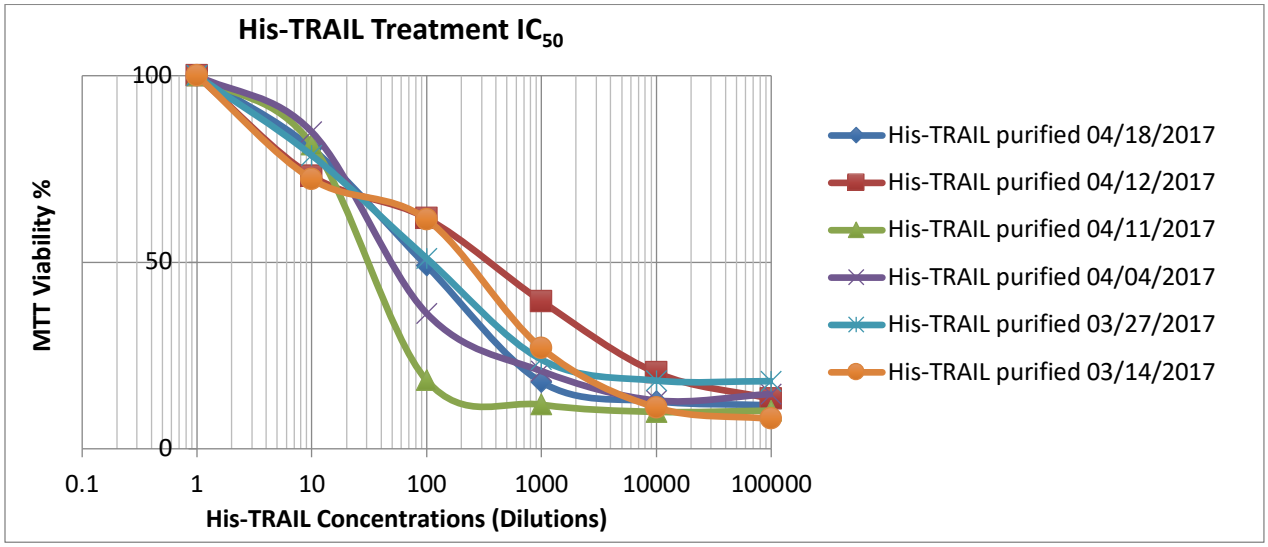


Figure 8 – Cell viability curve of U2OS cells treated with each hrTRAIL collection at the indicated concentrations along with 5µg/mL cycloheximide.

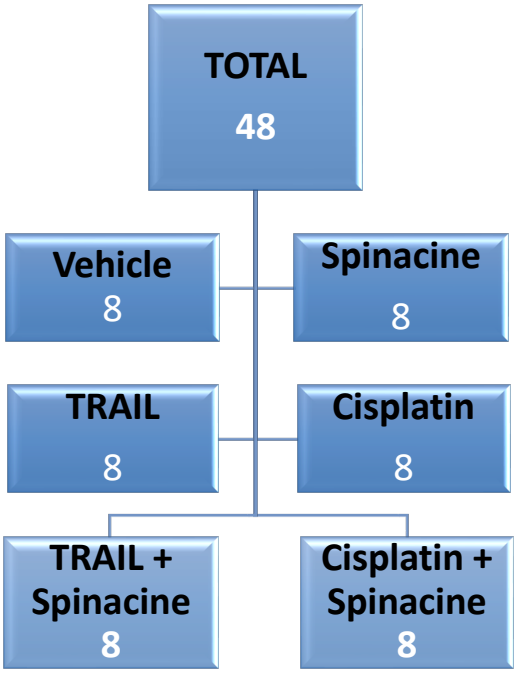
His-TRAIL Purified Dates	Average Concentration (mg/mL)	Dilutions @ IC_{50}	IC_{50} (mg/mL)	IC_{50} (ug/L)	Volume (mL)	Total Protein (mg)
03/14/2017	0.4672	87,000	5.37×10^{-6}	5.37	7	3.27
03/27/2017	0.6792	102,000	6.65×10^{-6}	6.65	9	6.11
04/04/2017	0.6561	600,000	1.09×10^{-6}	1.09	20	13.12
04/11/2017	0.6657	800,000	8.32×10^{-7}	0.83	12	7.99
04/12/2017	0.1819	73,000	2.49×10^{-6}	2.49	15	2.73
04/18/2017	0.8725	100,000	8.73×10^{-6}	8.73	26	22.68
				TOTAL	89	55.91

Table 1 – Protein concentration and IC_{50} of each hrTRAIL produced and collected.

2.2c) Spinacine antitumor efficacy *in vivo* study. To evaluate the ability of spinacine to synergize with hrTRAIL and cisplatin *in vivo*, we utilized a xenograft model from UM-SCC47 cells for HN cancer. For each model, spinacine were paired with both hrTRAIL and with cisplatin. Six groups of animals, with eight animals per group, were employed (**Figure 9a)**. Treatment strategy was outlined in the schematic shown in **Figure 9b-c**. On day 1, spinacine (5mg/kg) or vehicle was administered; and on day 2, it was applied in combination with an injection of either hrTRAIL (10 mg/kg) or cisplatin (5 mg/kg) (Duiker et al., 2009). hrTRAIL and cisplatin was administered intraperitoneally, and spinacine or vehicle injected directly into the tumor. After one day of rest, the same series of treatments was repeated 4 more times in a period of 2 ½ weeks (for a total of 5 treatments) after the initial treatment. Tumor size was monitored every 3-4 days with vernier caliper measurements. Tumor growth was corroborated with bioluminescent tumor imaging **(Figure 10b)**. The endpoint of the study was 120 days after tumor injection, and in some cases earlier, if signs of endpoints ~~pain and suffering~~ were observed.

Interestingly, the tumor growth curve demonstrated that the group treated with Spinacine in combination with TRAIL had the highest tumor increase, followed by vehicle; whereas the group treated with single-agent cisplatin showed the least growth **(Figure 10a)**. The combination treatment unfortunately did not amplify the effect of the apoptotic agents, in comparison to the single treatment groups. The IVIS imaging analysis also supported this finding **(Figure 10b-c)**. Furthermore, we analyzed the results by looking at the tumors that either increased (in green bars), did not change or decreased (in red bars), or regressed (shown in blue bars) *before and after* the treatment period **(Figure 11a)**. In the vehicle and the spinacine groups, the results demonstrated that most of the tumors grew and some of them regressed **(Figure 11b)**. Additionally, these two groups displayed the highest level of increasing tumor size according to the green bars, and the smallest number of decreasing tumors based on the red. The combined treatments demonstrated the lowest level of increasing tumor growth, and comparably the largest number of decreasing tumors compared to its single agent treated control groups. When the animals were treated with Cisplatin more tumors regressed. But when we added spinacine in the combination, we did not observe any further benefit. Therefore, we concluded that spinacine had no significant effect on the apoptotic agent efficacy in HPV^+ tumor growth *in vivo*.

A)



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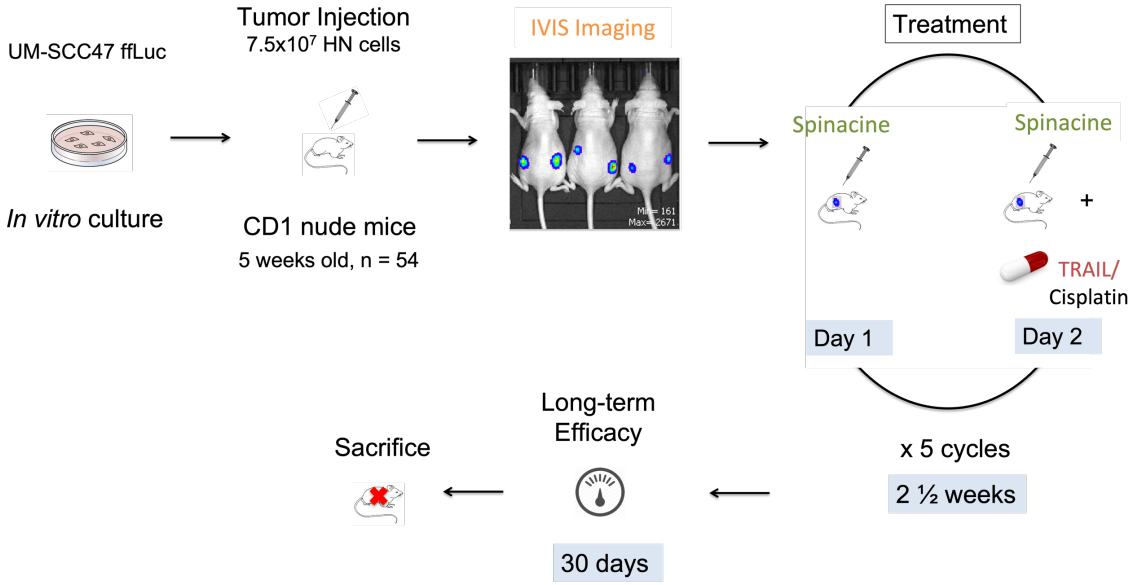
Study Design:



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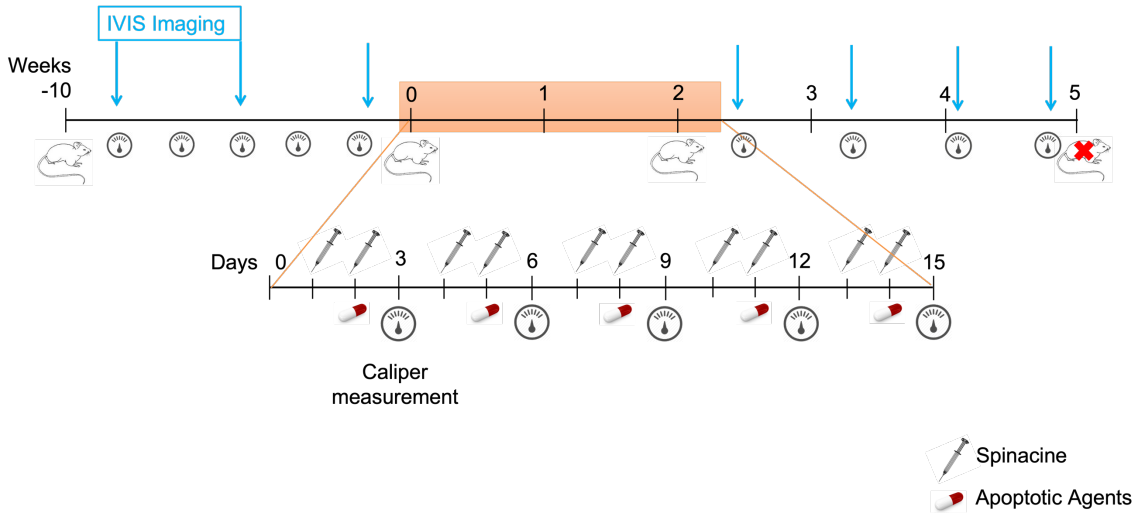
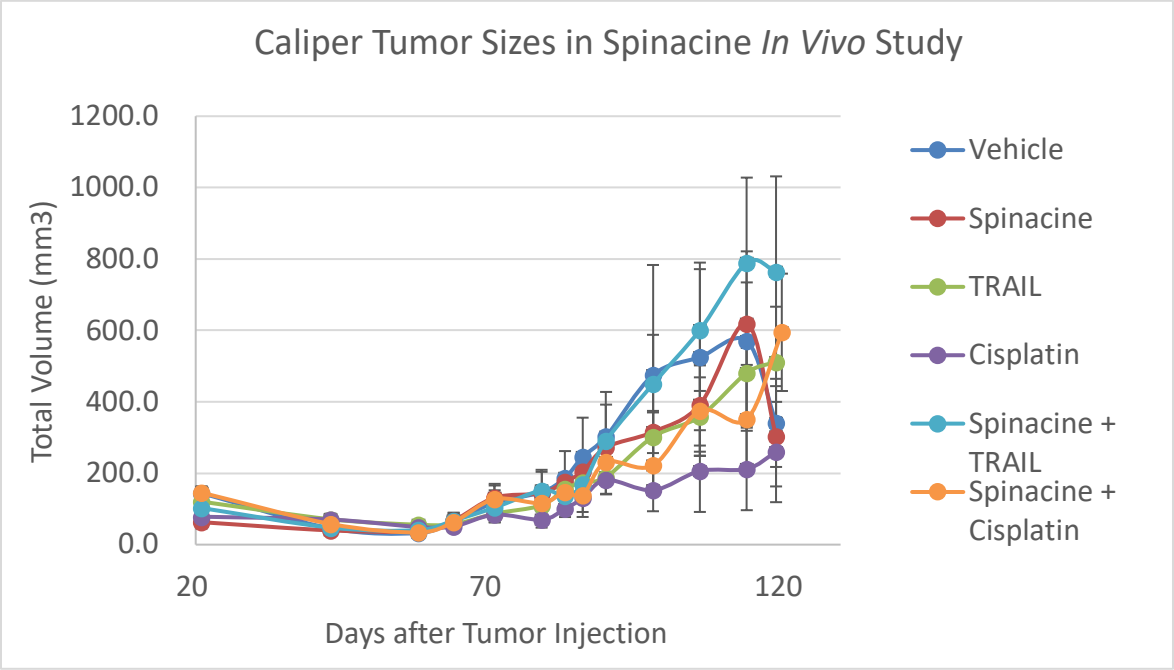
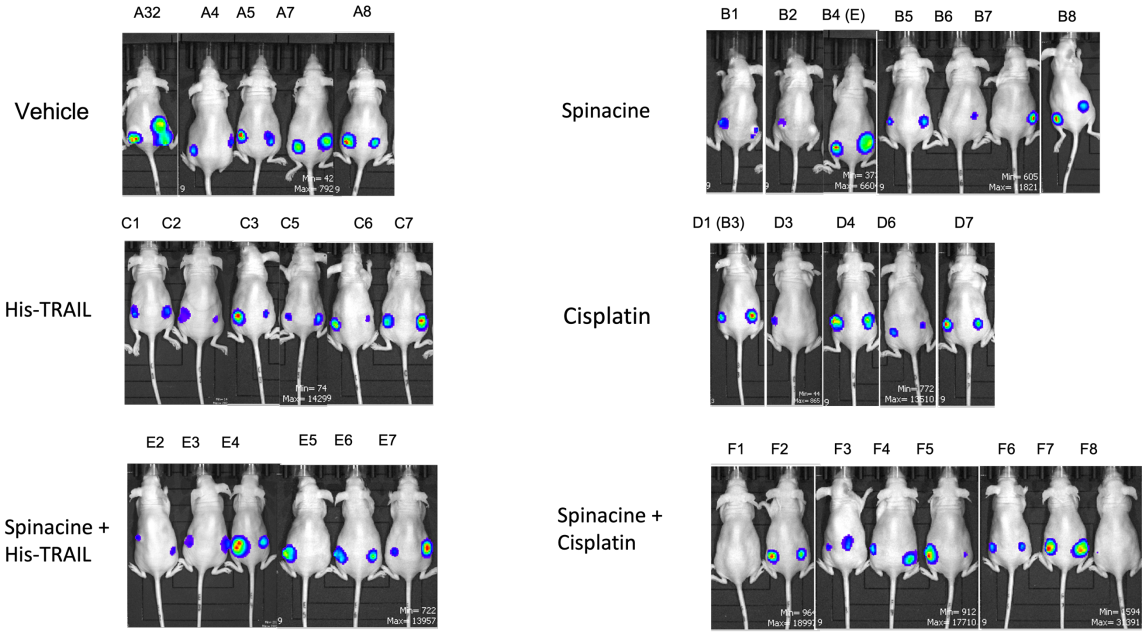


Figure 9 – Scheme of animal experiments **A)** Group of treatments. **B)** Study design. **C)** Scheme of treatment timeline.

A)


Caliper Tumor Sizes in Spinacine *In Vivo* Study
Total Volume (mm3)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
20
70
120
Days after Tumor Injection
Vehicle
Spinacine
TRAIL
Cisplatin
Spinacine + TRAIL
Spinacine + Cisplatin

B)



C)

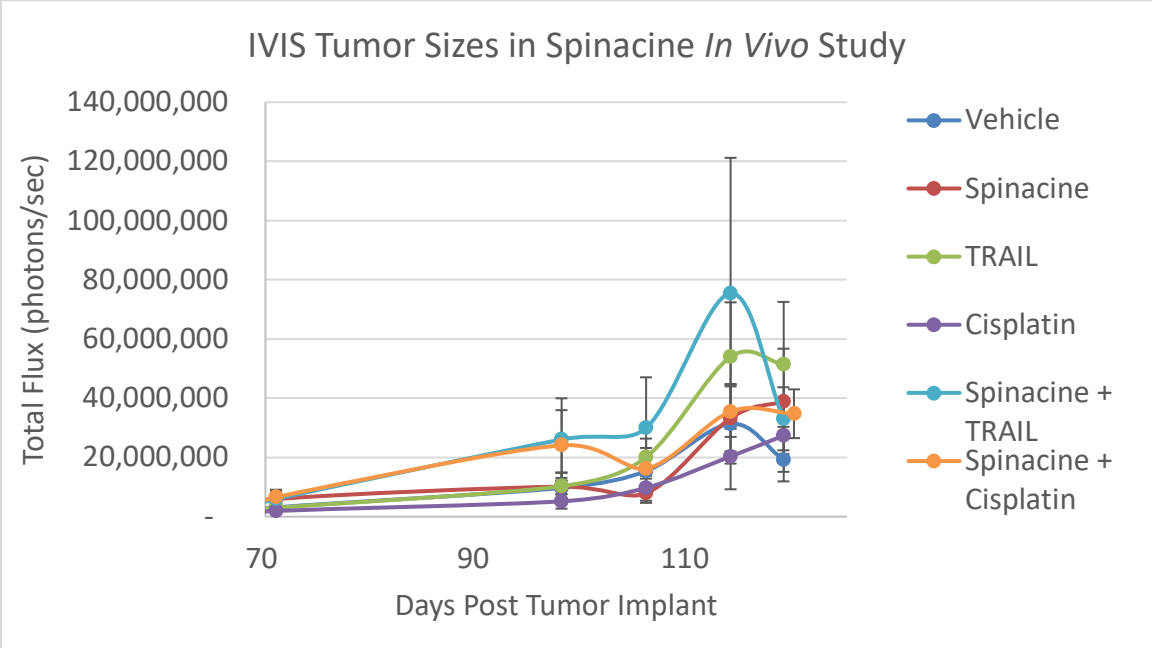
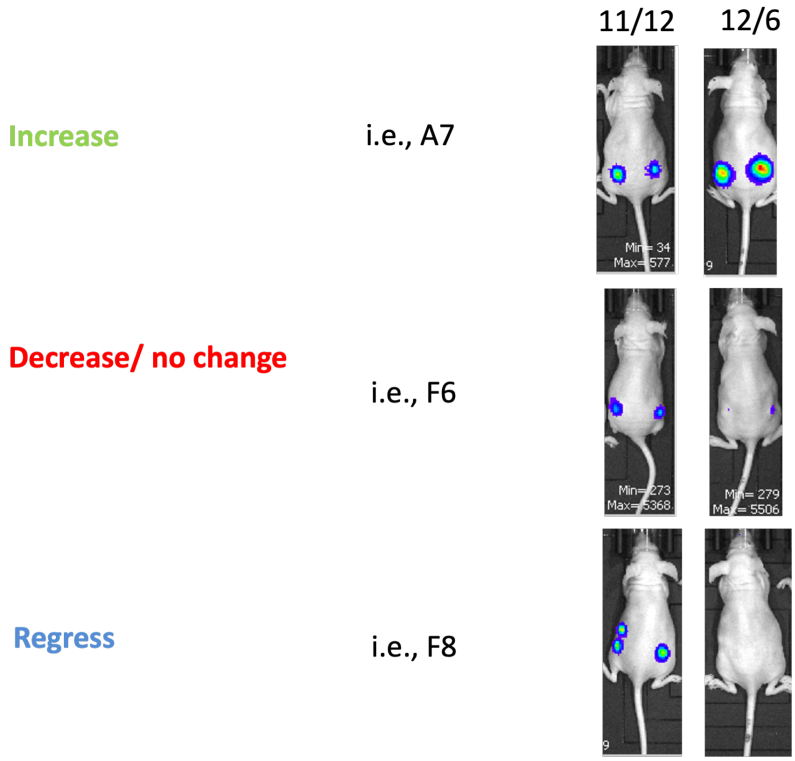


Figure 10 – Spinacine Tumor Growth Curve **A)** Caliper measurements of tumor volumes (mm^3). **B)** IVIS Images of treatment groups. **(C)** Bioluminescence intensity measurements of tumor sizes (in photons/sec).

A)



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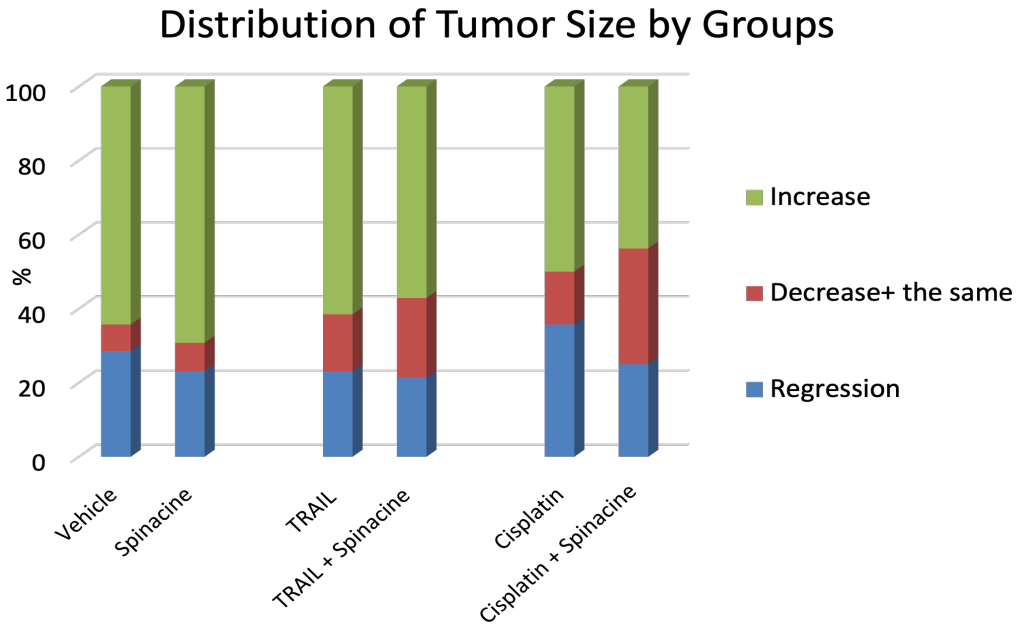

Figure 11 Tumor relative sizes before and after treatments A) Photo and diagrams of before and after mice tumor sizes. B) Percent distribution of mice tumor sizes after treatment relative to before treatment started.

3. Discussion

Previously, our laboratory had identified and tested small molecules that block E6/caspase 8 interactions, and our preliminary studies provided proof-of principle that molecules that block E6/caspase 8 interactions could, indeed, re-sensitize HPV^+ cells to apoptotic triggers (Tungteakkhun et al., 2010; Tungteakkhun et al., 2008; Yuan, Filippova, Tungteakkhun, et al., 2012). We found that the small molecule spinacine also demonstrated reconstitution of the cellular levels of caspase 8, FADD and p53 while it sensitized HPV^+ cells to apoptosis triggered by agents such as TRAIL or cisplatin in the cell context (Yuan et al., 2016; Yuan, Filippova, Tungteakkhun, et al., 2012). Therefore, we have successfully shown spinacine to interact with E6 *in vitro*; however, our first attempt at demonstrating this interaction *in vivo* did not yield evidence of efficacy. Mouse groups treated with cisplatin and in combination with spinacine demonstrated a decrease in tumor size; however, spinacine did not seem to amplify the antitumor effect of cisplatin *in vivo*. Therefore, we were unable to conclude that spinacine significantly increased chemotherapy efficacy in an HPV^+ HNSCC xenograft model. Many compounds show efficacy during initial studies *in vitro* and in cells, but not *in vivo*, and this efficacy study represented another such case. These divergent results could have been due to multiple metabolic processes and other complex biological effects present in an animal model. The impact of drug absorption, distribution, metabolism, and excretion could have potentially hampered the effectiveness of spinacine in the murine model. Other mechanisms of action that could have restricted spinacine's efficacy could be an inability of the compound to reach the target tumor cells and molecules within the animal. Additionally, due to the

use of several different lots of the compound, batch-to-batch variability could have affected the efficacy and outcomes of spinacine from the cellular context to the animal model. Notwithstanding, we have expanded the drug discovery strategy to include an *in vivo* model in the preclinical lead validation stage, and we have inched closer to translation by testing drugs through a more complex and representative system.

Combination therapies that can increase efficacy and decrease side effects in patients continue to be a desirable strategy. In practice, approaches based on cisplatin, carboplatin, paclitaxel, 5-fluorouracil, and radiotherapy are frequently combined into regimens drawing on two or even three agents; however, these combinatorial treatments have had limited efficacy and relatively serious side effects (Oikonomou & Pintzas, 2013; Stuckey & Shah, 2013). In contrast, the small molecule compound spinacine was shown to have low toxicity *in vivo*, as did TRAIL. Even though the combination of spinacine with the apoptotic-inducing agents did not show to be as effective as we expected, a decrease in growing tumors and an increase in tumors that shrank or stayed the same size was apparent **(Figure 8d)**. A more effective combinatorial approach could enable a reduction in the dosage, and thus, the side effects of some chemotherapeutic drugs. Therefore, combinatorial treatment plans with efficacious and low toxic small molecules that can deliver lower side effects in overall therapy are an important aspect in the context of HPV-induced head and neck carcinoma. Consequently, the concept that a small drug-like molecule in combination with other apoptosis-inducing agents or targeted therapy could provide safer and more effective therapeutic options to patients with HPV^+ malignancies, continues to be an approach still worth pursuing to improve clinical outcomes for these

patients. Even though our first study outcomes did not yield significant results, we still believe that finding other low toxic agents that target HPV E6 or other oncoproteins would be an excellent therapeutic strategy to treat HPV-associated cancers; and small molecule drugs could also prove to be an appropriate approach to reach this goal.

In this study, a xenograft model has been developed, optimized, and tested for lead molecule validation. The extended murine xenograft model included the growth of HPV^+ HN tumors in addition to the established HPV^+ cervical cell line. Moreover, the optimized HPV^+ HN cancer mouse model incorporated the use of a basement membrane matrix and luciferase reporter technology for a more consistent tumor growth and state-of-the-art tumor visualization in a real-time manner, respectively. The enhanced xenograft model enabled us to evaluate the antitumor efficacy of a small molecule, spinacine, and to observe its effect on the growth of HN-derived tumors both through Vernier caliper and BLI measurements. Thus, we have optimized the *in vivo* xenograft animal model for preclinical lead validation of any additional E6 inhibitors that may be found in the future. The optimized and tested *in vivo* model will put us in a good position to evaluate any new leads we may obtain for potential therapeutic use. In conclusion, we have developed an *in vivo* model for preclinical lead validation on future small molecule candidates that could potentially be even more potent E6 inhibitors. Consequently, this could generate future combination therapies that can attenuate toxicity and therapeutic sequelae while enhancing treatment efficacy and improving patients' clinical outcomes.

4. Material and Methods

Cell Culture

HPV^{+} HNSCC cell lines were obtained from several sources: UD-SCC-2 Clone 5; UPCI:SCC90 Clone 35; UM-SCC47 Clone 3; 93-VU-147T Clone 6; were a gift from Dr. John Lee, Sanford Research (South Dakota, USA). UM-SCC47 and UM-SCC-104 were a gift from Dr. Thomas Carey, University of Michigan (Michigan, USA) (Tang et al., 2012). All HN cells were cultured in Dulbecco's Modified Eagle Medium (Mediatech, Manassas, VA, USA) supplemented with 9% fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Sigma Aldrich). U2OS cells were obtained from ATCC (Manassas, VA) and were grown in McCoy's media supplemented to 10% FBS (Invitrogen), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Sigma Aldrich).

Reagents

Spinacine, also known as 4,5,6,7-tetrahydro-1H-imidazo[4,5–c]pyridine-6-carboxylic acid, was provided by TimTec LLC (Newark, DE, USA) and dissolved in DMSO to yield a 200 mM stock. Cisplatin (Accord Healthcare Inc, Durham, NC) was a donation from Dr. Kofi Donkor and his pharmaceutical team at the Loma Linda University Medical Center Cancer Center. Matrigel (Corning Inc, Somerville, MA), a basement membrane mixture, was administered to facilitate the engraftment and implantation of the tumor grafts. Saline solution (0.9%) was used for vehicle treatment.

hrTRAIL Protein Purification & Cell Viability Assay

We cloned the extracellular C-terminal domain of human TRAIL into the protein expression vector *pTriEx-4* with an N-terminal (His-)$_6$ tag. The plasmid pTriEx-4 His-TRAIL was expressed in *E. coli BL-21 pLys* cells and induced with IPTG to produce His-TRAIL protein. Then, His-tagged human recombinant TRAIL (hrTRAIL) proteins were resuspended in His dilution buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 300uM imidazole, 2 μM DTT). The collected hrTRAIL concentration was determined using Coomassie Plus – The Better Bradford Assay Reagent (Thermo Scientific, Waltham, MA). Isolated hrTRAIL was separated by gel electrophoresis (SDS-PAGE) and purity determined by Coomassie staining. To measure cell viability following hrTRAIL treatment, a TRAIL-sensitive cell line, U2OS, was seeded into 96-well plates (1-2 × 10^4 cells/well) and allowed to adhere overnight. The treatment of U2OS cells with hrTRAIL was added in the presence of cycloheximide (5 μg/ml) to inhibit *de novo* protein synthesis, and the cells were incubated at 37^{o}C for 16 hours prior to measuring cell viability by the MTT assay**.** Twenty microliters of MTT were added (10 mg/ml stock), and cells were incubated at 37 °C for 2-3 hours. The media was removed, and 100-150 μl of DMSO was added and mixed with MTT, turning the solvent into a purple hue. The absorbance of each well was measured at 490 nm.

Transduction of Luciferase expressing cells

To allow tumor *in vivo* visualization, UM-SCC47 cells were transduced with a CMV-p:EGFP-*ffluc* pHIV7 lentiviral vector (Brown et al., 2009), a kind gift from Dr. Juli Unternaehrer at Loma Linda University (Loma Linda, CA USA). The lentiviral vector

encodes a fusion protein of enhanced green fluorescence protein (eGFP) and *firefly luciferase* (*ffLuc*). The eGFP-*ffLuc*-transduced UM-SCC47 cells were selected based on *firefly luciferase* expression via the SpectraMax i3x microplate reader (San Jose, CA). The UM-SCC47 eGFP-*ffLuc* cells that passed primary screening with high bioluminescence intensity (BLI) underwent secondary screening (**Figure 6A**). UM-SCC47 *ffLuc*-eGFP Pool 1 cells demonstrated to have the highest luciferase expression (**Figure 6B**) and hence we selected them to initiate tumors in our *in vivo* model.

Animal Experiments

All animal experiments were approved and carried out in accordance with the Institutional Animal Care and Use Committee at Loma Linda University and the Loma Linda VA Medical Center (IACUC# 888, 8140036, 8140045, 8170026). 5 weeks old female CD-1 (Crl:CD1-Foxn1nu-086) immunodeficient nude mice obtained from Charles River Laboratories (Wilmington, MA) were used as a xenograft model in most of the studies performed. Experiments that used athymic nude mice were conducted under sterile conditions at the animal research facilities (Loma Linda, CA). Animal procedures were performed under 3% inhaled isoflurane. Sterilized food and water were provided ad libitum. Animals that exhibited signs of pain or distress or other endpoint criteria were euthanized.

To establish an HPV^+ HNSCC xenograft model in **Section 2.1a**, $7.5x10^6$ cells of six HPV^+ HN cell lines (UD-SCC-2 Clone 5; UPCI:SCC90 Clone 35; UM-SCC47; UM-SCC47 Clone 3; 93-VU-147T Clone 6; UM-SCC-104) were injected intradermally into both flanks of

athymic mice. Two mice were assigned per cell line, for a total of 12 animals. Tumor growth over time were monitored by Vernier caliper measurements.

For more consistent tumor growth, the basement membrane Matrigel was tested with two different HPV^+ HN cell lines (UPCI:SCC90 Clone 35 and UM-SCC47) in **Section 2.1b**. Three nude mice were injected bilaterally with 1-1.5 x 10^7 tumor cells with Matrigel in a 1:1 ratio in group 1, whereas group 2 did not contain any Matrigel as the control group. The tumor volumes of both groups were measured with calipers and then compared over time.

For tumor *in vivo* visualization in **Section 2.1c**, mice were injected with 10^7 UM-SCC47 *ffLuc*-transduced Pool 1 cells subcutaneously on both flanks of each athymic mouse. BLI measurements of tumor growth and metastasis development were performed every week after intraperitoneal injection of the luciferase substrate GoldBio luciferin potassium salts (GoldBio) (2 mg in 200µl). Ten minutes following injection, the fully anesthetized animal was placed into the imaging chamber, and the image was acquired using the IVIS Lumina III In Vivo Imaging System (PerkinElmer, Waltham, MA) **(Figure 6C)**. Tumor growth was measured using the Living Image software (PerkinElmer).

The dose-finding study in **Section 2.2a** tested 3 different concentrations of spinacine (vehicle, 5mg/kg, 10mg/kg, 20mg/kg) on CD-1 female mice (Crl:CD-1-022) that were 26-30 days old. Saline solution was utilized in the vehicle groups. Three mice were randomly assigned into each of the four groups based on body weight. No tumor engraftments or athymic nude mice were used in this study. Clinical observations including physical, behavioral assessments and weight were monitored throughout the experiment,

especially during and after spinacine treatment. Blood was collected through cardiac puncture right after euthanasia, and major internal organs were evaluated and harvested post-mortem.

In the tumor inhibition study in **Section 2.2c**, the xenograft model was established by subcutaneous injections of 7.5 x 10^6 - 10^7 UM-SCC47 *ffLuc* eGFP Pool 1 cells in 50% Matrigel into the flanks of nude mice. Eight mice were randomly assigned to each experimental group: A) Vehicle B) Spinacine C) hrTRAIL D) Cisplatin E) hrTRAIL + Spinacine F) Cisplatin + Spinacine. Saline treatment was utilized in the vehicle and single-treatment control groups. The number of mice per group was estimated based on our preliminary experiments with xenograft mice, so as to obtain a statistically significant difference in tumor growth between control and tested groups. Power calculations indicate that in order to detect a difference of at least 25% in mean tumor volume when administering the therapeutic agent, with a power of 90% and an alpha (Type I) value of 0.05, 8 mice will be necessary for each group (8 mice x 6 groups = 48 mice/experiment). Animals bearing tumors will be randomly assigned into the various treatment groups based on body weight and tumor size. Tumors were grown for 10 weeks before the volumes reached 100 mm^3 and treatments were started. Tumor volumes were measured until the tumor reached 5000 mm^3 in size, at which point the animal was euthanized by CO_2 asphyxiation and exsanguination through cardiac puncture. Tumor growth was monitored through Vernier caliper measurements and IVIS imaging, then the tumors were harvested after sacrifice.

Tumor growth analysis

Tumors were dissected, photographed near a ruler (for size estimation) and weighed. Tumor volume was determined twice a week by Vernier caliper measurements. The length and width of each tumor was measured, and tumor volumes (V) were calculated using the following formula: $V=LW^2/2$. The length (L) was considered as the longest diameter, whereas the width (W) was determined as the maximum diameter perpendicular to the direction of the longest diameter measured by the vernier.

Moreover, tumor bearing mice were imaged using a cooled charge-coupled device camera system from IVIS Lumina III (Perkin-Elmer, Waltham, MA). After being anesthetized, 150uL of 10mg/mL D-Luciferin potassium salts (GoldBio, St. Louis, MO) were injected intraperitoneally into the abdominal cavity of the animals. Mice were placed in the light-tight chamber of the imaging system under isoflurane anesthesia and imaged 5 min after injection. We acquired sequences of 9 images with binning of 2, 4, and 8 and at exposure times 5, 30, 60 seconds, with a preset condition of F/stop of 1 and a field of view of 12.5. Once the images were captured, regions of interest (ROI) were placed over the tumors to quantify the signal intensity within those regions using the Living Image Software (Caliper Life Sciences, Alameda, CA). BLI detected was represented as a total flux measurement in photons/sec.

Statistical analysis

Data on tumor growth will be presented as a group average in mm^3 or photons/sec at each time point +/- standard error (SE). Within each group, tumor size at a certain time point will be expressed as a percentage of tumor size at the treatment inception.

CHAPTER THREE

30-HYDROXYGAMBOGIC ACID INCREASES CISPLATIN EFFICACY IN AN HPV^{+} HNSCC XENOGRAFT MODEL

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, affecting more than half a million people annually (cancer.net). HNSCC is an epithelial tumor arising in the mucosal lining of the oral cavity, nasal cavity, oropharynx, larynx, and hypopharynx. The traditional etiology for HNSCC is the use of tobacco and alcohol; however, a steady HNSCC rise in developed countries in the past couple of decades has established high-risk human papillomavirus (HR-HPV) as a risk factor (Sabatini & Chiocca, 2020; Scott-Wittenborn et al., 2022; Ursu et al., 2022). Nearly 70% of oropharyngeal cancer are associated with HPV infection, and HPV type 16 accounting for 82% of HPV positive HNSCC (Alsbeih et al., 2019; Cavallo, 2021; Ferris & Westra, 2023; Ghosh, Shah, & Johnson, 2022; Johnson et al., 2020; Mahal et al., 2019; Ndiaye et al., 2014; Ursu et al., 2022; Ward, Mehta, & Moore, 2016). Gardasil 9 is a vaccine approved in 2020 for preventing several HPV-malignancies including HNSCC (Diana & Corica, 2021; Liao et al., 2022; J. Z. Zhou, Jou, & Cohen, 2021). Unfortunately, these prophylactic vaccines do not benefit people already infected with HPV or with a compromised immune system.

Once tumors develop, current therapeutic approaches for HNSCC are limited and are not optimally customized for the evolving subtypes of HNSCC. Each subset of HNSCC is characteristically different and exhibits divergent clinical outcomes, as HPV-infected patients experience better cancer treatment response and survival than their non-HPV counterparts (Sabatini & Chiocca, 2020). Current treatment guidelines do not distinguish between HPV status, and standard of care consists of invasive surgery, chemotherapy, radiation, or a combination of these modalities (Ursu et al., 2022). The challenge with

HPV$^+$ HNSCC patients is the resulting post-treatment long-term morbidity rather than treatment efficacy, which is typically quite high (82%) (Sun, Wang, Qiu, & Wang, 2021). The classical therapeutic regimen used to treat HNSCC patients leads to long-term sequelae in the otherwise high functioning nonsmoking young male population, which is the representative demographic of this cancer subtype (Amjad, Chidharla, & Kasi, 2023; Meistrich, 2013). Therefore, treatment side-effects frequently create functional complications, such as difficulties and impairments with chewing, swallowing, and breathing; these sequelae compromise a young patient's quality of life with decades left to live (Ghosh et al., 2022; Mott et al., 2020; Riechelmann et al., 2022). Due to the myriad of toxicities associated with chemotherapy, targeted therapy has become an attractive treatment strategy due to its typically favorable side effect profile and increased efficacy. To date, cetuximab is the only targeted therapy that is widely used for HNSCC (Li, Tie, Alu, Ma, & Shi, 2023). That said, cisplatin, a cytotoxic agent and a DNA intercalator, remains the standard of care for HNSCC. Therefore, new improved treatment approaches that can lower toxicity while specifically targeting the tumor must be developed.

To increase the effectiveness while decreasing the toxicities of current treatment regimens, our lab screened and tested several small molecule libraries, leading to the identification of 30-hydroxygambogic acid (GA-OH) as an inhibitor of E6 (Chitsike, Yuan, Roy, Boyle, & Duerksen-Hughes, 2021). The oncoprotein E6 of HR-HPV has been identified as a major contributor to the oncogenicity of HPV. E6 blocks multiple apoptotic pathways, mainly through the degradation of proteins such as p53 and caspase 8, thereby allowing the virus to persist in the host and increasing the probability of tumor formation. The small

molecule GA-OH is able to selectively inhibit E6 activity by binding to the oncoprotein, which increases the levels of p53, caspase 8, p21 and caspase 3 (Chitsike et al., 2021), thereby sensitizing tumor cells to chemotherapy-induced apoptosis.

The subsequent step in our research was to make the transition to animal models and test the effectiveness of the application of GA-OH alone and in combination with chemotherapy *in vivo*, using cisplatin and cetuximab as model agents. Our hypothesis is that due to its ability to inhibit E6 and sensitize HPV^+ cells to apoptosis, GA-OH will increase the effectiveness of chemotherapeutic agents such as cisplatin. We plan to test the ability of GA-OH to reduce or eliminate tumor growth in a mouse xenograft model, along with a GA-OH dose-finding study and toxicological assessments. Success in this study would advance the development of effective therapeutic approaches to combat HPV-associated malignancies.

Materials & Methods

Cell Culture

UM-SCC47 *ffLuc*-eGFP (Brown et al., 2009) Clone 14 is a stably transduced luciferase expressing variant of the human HPV^+ head and neck cancer cell line UM-SCC47 (al. 1979), which were a gift from Dr. Thomas Carrey, University of Michigan (Michigan, USA). The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (Genesee, San Diego, CA), supplemented with 9% fetal bovine serum (Genesee), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Sigma-Aldrich), and maintained at 37°C in a humidified atmosphere of 95% air / 5% CO_2.

Reagents

30-hydroxygambogic acid (GA-OH) obtained from Quality phytochemicals LLC (New Jersey, USA) was dissolved in DMSO to a stock solution of 6447.5 mg/L (10 mM) concentration before diluting it into a final concentration of 120 mg/L (186.119 μM) immediately prior to use in the tumor inhibition study. Cisplatin (Accord Healthcare Inc, Durham, NC) and cetuximab (Erbitux) (ImClone LLC, Branchburg, NJ) were a donation from Dr. Kofi Donkor and his pharmaceutical team at the Loma Linda University Medical Center Cancer Center. Cultrex (R&D Systems, Minneapolis, MN), a basement membrane mixture, was used for the engraftment and implantation of the tumor grafts. Sterile saline solutions (0.8%) were administered for vehicle treatment.

Transduction of Luciferase expressing cells and clone selection

Transduction of UM-SCC47 cells

UM-SCC47 cancer cells were transduced with a CMV-p:EGFP-*ffLuc* pHIV7 lentiviral vector (Brown et al., 2009), a kind gift from Dr Juli Unternaehrer at Loma Linda University (Loma Linda, CA USA). The lentiviral vector encodes a fusion protein of enhanced green fluorescence protein (eGFP) and *firefly luciferase* (*ffLuc*). Once cells were transduced, single cell clones were isolated by limiting dilution and expanded *in vitro.*

Flow cytometry: GFP expression

Cell populations arising from single-cell clones generated 39 stable clones. GFP levels of these clones were analyzed by flow cytometry (MACSQuant Analyzer 10, San Diego, CA). Cell populations were gated as either GFP^+ or GFP^- through the FL3A channel, and the percentages of GFP^+ cells determined. Each clone GFP measurement was repeated at least three different times (more than three biological replicates carried out on different days). Data represented are from a representative experiment.

In-Vitro IVIS Assay: Bioluminescence Detection

The specificity and sensitivity of luciferase expression was demonstrated by serial two-fold dilutions of UM-SCC47 clones. Serial dilutions starting with one million cells of luciferase-transduced UM-SCC47 cells were prepared by mixing 100 μL of tumor cells into 100 μL of DMEM media, resulting in a 2-fold dilution (1000000, 500000, 250000, 125000, 62500, 31250 cells per well). Once 100 μL of the clones were added onto a black 96 well

plate in a gradient of decreasing concentration, bioluminescence detection was conducted after adding 100 μL of 150 μg/mL of luciferase substrate into each well. We incubated the plate for 5-10 min before imaging using the IVIS Lumina III system (Perkin Elmer, Waltham, MA). The *in-vitro* assay was repeated at least three times (more than three biological replicates, carried out on different days). Data presented are from a representative experiment. Quantitative analysis was achieved by analyzing the correlation between the number of tumor cells and the intensity of the bioluminescence.

Dose-Finding Study

We induced HPV^+ HNSCC in mouse xenograft models by subcutaneously injecting 10^7 of UM-SCC47 *ffLuc*-eGFP clone #14 cells into CD-1 (Crl:CD1-Foxn1nu-086) immunodeficient nude mice. The mice received different dosages of GA-OH starting with 5 mg/kg and ranging all the way down to 0.25 mg/kg. 125 μL of the appropriate GA-OH dose or saline were delivered locally on Days 1 and 2. No injections were made on Day 3, and this 3-day cycle was repeated 5 times (for a total of about 2 ½ weeks). Following GA-OH injections, the spot of injection was inspected for redness, swelling, discharge, or other signs of toxicity. Mice were weighed regularly, and tumor growth measured by Vernier calipers and monitored by bioluminescence imaging. Mice were monitored for general health (hydration, mucus membranes, discharge, weight, mobility) throughout the experiments. If an animal exhibited signs of pain or distress, or their tumors reached 20 mm in one dimension, they were euthanized by carbon dioxide (CO_2) narcosis followed by exsanguination.

Tumor implantation and animal maintenance

The mice underwent subcutaneous injections of $1.5x10^7$ UM-SCC47 *ffLuc*-eGFP clone #14 cells to establish subcutaneous tumors in CD-1 (Crl:CD1-Foxn1nu-086) immunodeficient nude mice. 60 naïve 4-6 weeks old mice comprised of 30 female and 30 male mice were obtained from Charles River Laboratories (Wilmington, MA) and housed in pathogen-free conditions with individually ventilated cages. The tumor cells were resuspended in 100 μL DMEM and 100 μL Cultrex in a 1:1 ratio and implanted into the flanks using a 1 mL syringe.

Ten animals were randomly assigned to each experimental group based on tumor size and gender: A) Vehicle; B) GA-OH alone; C) Cisplatin alone; D) Cisplatin + GA-OH; E) Cetuximab alone; F) Cetuximab + GA-OH. The number of mice per group was estimated based on our preliminary experiments with xenograft mice, so as to obtain a statistically significant difference in tumor growth between control and tested groups. Power calculations indicate that to detect a difference of at least 35% in mean tumor volume when administering the therapeutic agent, with a power of 90% and an alpha (Type I) value of 0.05, 10 mice will be necessary for each group (10 mice x 6 groups = 60 mice/experiment).

The treatment regimen started a week after tumor injection with 120 mg/L GA-OH or saline direct intratumoral injections to each group. Saline was used as treatments in the vehicle and single-treatment control groups. The chemotherapeutic drugs cisplatin (1 mg/mL) or cetuximab (2 mg/mL) were administered intraperitoneally. Approximately 125 μL of each small molecule or chemotherapeutic agent were injected into each group,

using sterile techniques. An assessment such as tumor size, weight, level of activity, posture and changes in behavior were monitored every 2-3 days during the treatment period. We monitored the mice for another 3 weeks to assess the long-term efficacy of the treatments. If an animal exhibited signs of pain or distress or tumors larger than 20 mm in one dimension, they were euthanized earlier. Mice were sacrificed by CO_2 inhalation in a chamber, followed by exsanguination through cardiac puncture. Blood was collected for serum and hematological tests. Tumors were dissected, photographed near a ruler (for size estimation) and weighed. The following end point parameters were evaluated: mortality, clinical signs, body weight, hematology and serum biochemistry, and post-mortem major organ evaluation.

All animals were housed at the Loma Linda University animal care facility (ACF) and acclimated for at least one week before the start of the studies, and experimental procedures were conducted under sterile conditions. Standard irradiated chow diet and sterilized water were provided *ad libitum*. All animal procedures and protocols were reviewed and approved by the Loma Linda University Institutional Animal Care and Use Committee (IACUC 20-127, 8 March 2022).

Tumor growth measurements

Tumor volume was determined twice a week by Vernier caliper measurements (Carolina Biological Supply, Burlington, NC). The length and width of each tumor was measured, and tumor volumes (V) were calculated using the following formula: $V=LW^2/2$. The length (L) was considered as the longest diameter, whereas the width (W) was determined as the maximum diameter perpendicular to the direction of the longest diameter measured by the vernier.

Moreover, tumor bearing mice were imaged using a cooled charge-coupled device camera system from IVIS Lumina III (Perkin-Elmer, Waltham, MA). After being anesthetized, 150 μL of 40 mg/mL D-Luciferin potassium salts (GoldBio, St. Louis, MO) were injected intraperitoneally into the abdominal cavity of the animals and the mice then placed in the isoflurane induction chamber 5 min after substrate injection. Once under anesthesia, mice were transferred to the light-tight chamber of the imaging system and imaged 13 min after substrate administration. We acquired sequences of 37 images with binning of 2, 4, and 8 and at exposure times of Auto, 0.5, 1, 2, 4, 10, 20 seconds, with a preset condition of F/stop of 1 and a field of view of 12.5 for every batch. Once the images were captured, regions of interest (ROI) were placed over the tumor images to quantify the signal intensity within those regions using the Living Image Software (Caliper Life Sciences, Alameda, CA). BLI detected was represented as a total flux measurement in photons/sec.

BLI Kinetics of Tumor model

Bioluminescence kinetic measurements were determined in our xenograft model to ensure maximum and consistent photon flux. IVIS images were acquired starting 8 min after substrate administration and continued until 24 min after. Sequence acquisitions were made every 2 min per batch. Sequential images were set at binning of 4 and Auto exposure time, with a preset condition of F/stop = 1, and a FOV of 12.5. BLI were measured by Living Image Software as described above. Each kinetic measurement was repeated at least three different times (more than three biological replicates carried out on different days). Data represented are from a representative experiment.

Statistical analysis

Data on tumor growth is presented as a group average in mm^3 at each time point +/- standard error (SE). Within each group, tumor size at a certain time point is expressed as a percentage of tumor size at the treatment inception. One-way ANOVA was used to determine statistical significance of any observed differences between groups regarding end-point organ weight, tumor weight, and any blood test and toxicity panel analysis. Statistics were calculated using Prism software 9.4.1 (Graphpad Software Inc, San Diego, CA).

Generalized Least Squares Regression (GLSR) was used to assess the effects of group assignment, time, and group x time interaction on tumor size. Three group comparisons were made: 1. Cisplatin + GA-OH compared to Vehicle, 2. Cisplatin + GA-OH compared to GA-OH, and 3. Cisplatin + GA-OH compared to Cisplatin. Data analysis was

performed using R (version 2022.07.2). Generalized least squares regression (GLSR) was used to compare differences in tumor growth over time between groups. GLSR assumptions were checked, including homoscedasticity and normality of residuals, to ensure the reliability of the results. A *p*-value of < 0.05 was considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

All *in vitro* experiments were repeated at least three times (three biological replicates, carried out on different days), with each experimental group measured in triplicate within each of these individual experiments. Data presented are from a representative experiment.

Results

Clonal Selection:

To allow for *in vivo* visualization, tumor cells expressing the *Luciferase* were injected subcutaneously in nude mice to allow engraftment and tumor formation. The UM-SCC47 EGFP-*ffLuc* stable clone cell line was used to produce the head and neck cancer mouse xenograft. We began by transducing the HPV^+ HN cancer cell, UM-SCC47, with *firefly luciferase*. We used the lentiviral vector CMV-p:EGFP-*ffLuc* pHIV7, which contains genes coding for both green fluorescence protein (GFP) and luciferase (*ffLuc*) **(Figure 12)**. Once transduced, we isolated and expanded single cell clones, generating 39 stable clones. We tested all clones through flow cytometry for levels of GFP expression **(Table 2)**. Only eight of the 39 clones demonstrated a fluorescence intensity of 50% or higher in the primary screening. After secondary screening **(Figure 13a-b)**, the scatter plot and

histograms consistently indicated clones #14, #31, and #35 to have the highest percentages of GFP^+ cells (greater than 98%) **(Table 3)**. When we tested the levels of *firefly luciferase* (*ffLuc*) expression by serial dilutions through IVIS imaging, clone #14 exhibited a higher level of bioluminescence signal **(Figure 14a-b)** compared to the other highly GFP-expressing clones. The minimum number of Clone 14 cells that was reliably and quantitatively detected *in vitro* with this system was 10^5 cells/well using a 96-well black plate with a final luciferase concentration of 75 μg/ml (total 200uL). Moreover, we found that the BLI signal was proportional to the absolute amount of plated clonal cells. A strong linear correlation ($R^2 = 0.995$) was observed between absolute cell number and BLI signal. **(Figure 14c)**. In conclusion, clone #14 cells had the highest level of GFP and *firefly luciferase* and was selected to initiate tumors in our xenograft model.

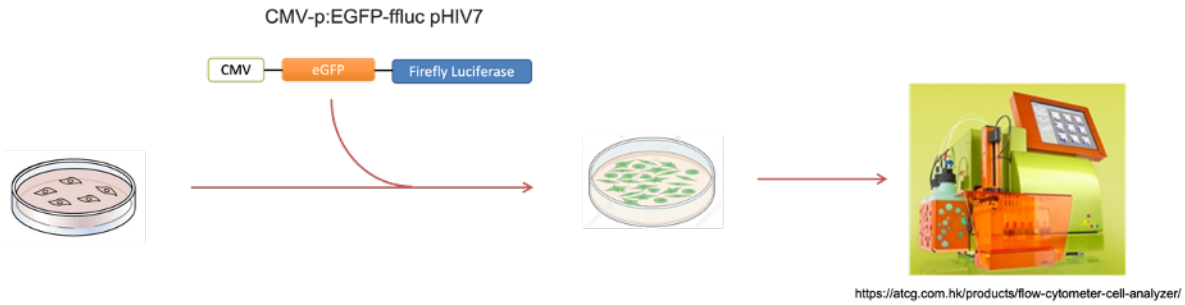
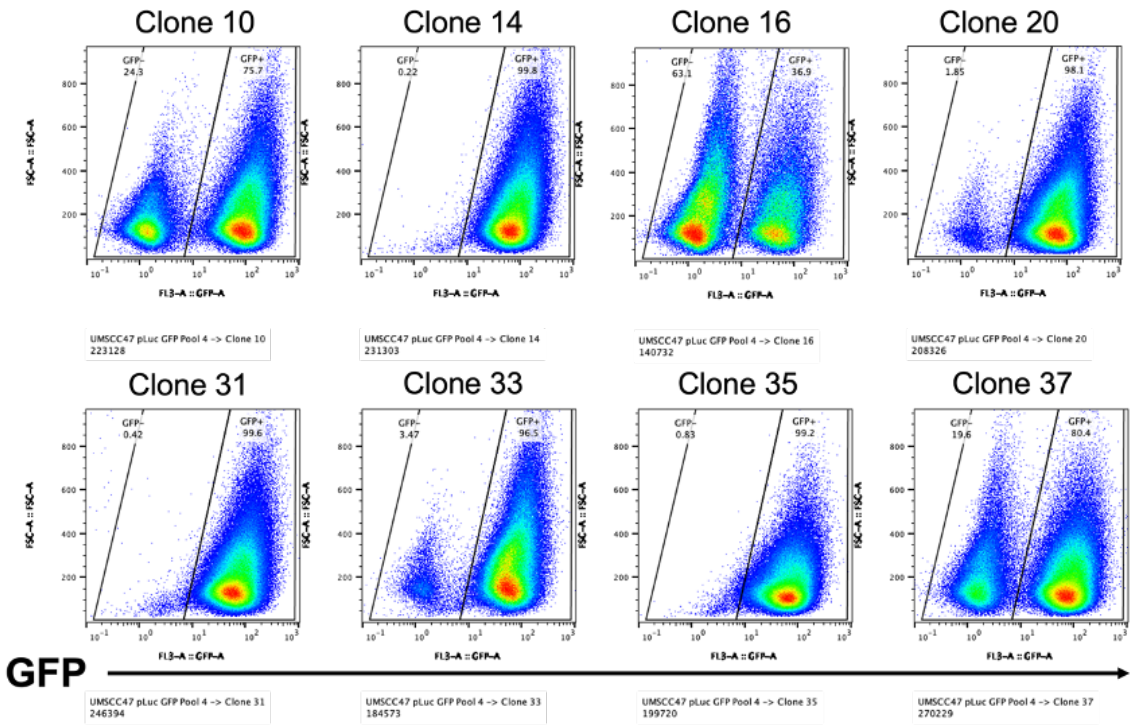


Figure 12 – Transduction of UM-SCC47 cells with lentiviral vector CMV-p:EGFP-*ffLuc* pHIV7, which expresses *firefly luciferase* (*ffLuc*) and green fluorescent protein (GFP). Partial clonal selection was based on GFP expression by flow cytometry.

CLONES	GFP %	CLONES	GFP %	CLONES	GFP %
1	2.26	15	12.05	33	95.37
2	1.24	16	57.26	34	30.30
3	4.06	17	0.52	35	98.29
4	0.35	18	6.33	36	0.25
5	14.00	19	0.28	37	80.52
6	0.21	20	97.04	38	7.16
7	1.19	22	5.85	41	36.63
8	1.85	23	2.80	42	0.35
9	0.12	24	3.15	43	1.35
10	70.40	29	0.43	44	0.21
12	0.12	30	0.78	45	0.38
13	1.30	31	98.75	46	29.50
14	98.96	32	0.50	47	1.27

Table 2 – Percentages of GFP^+ populations in all stable clones generated.

A)



B)

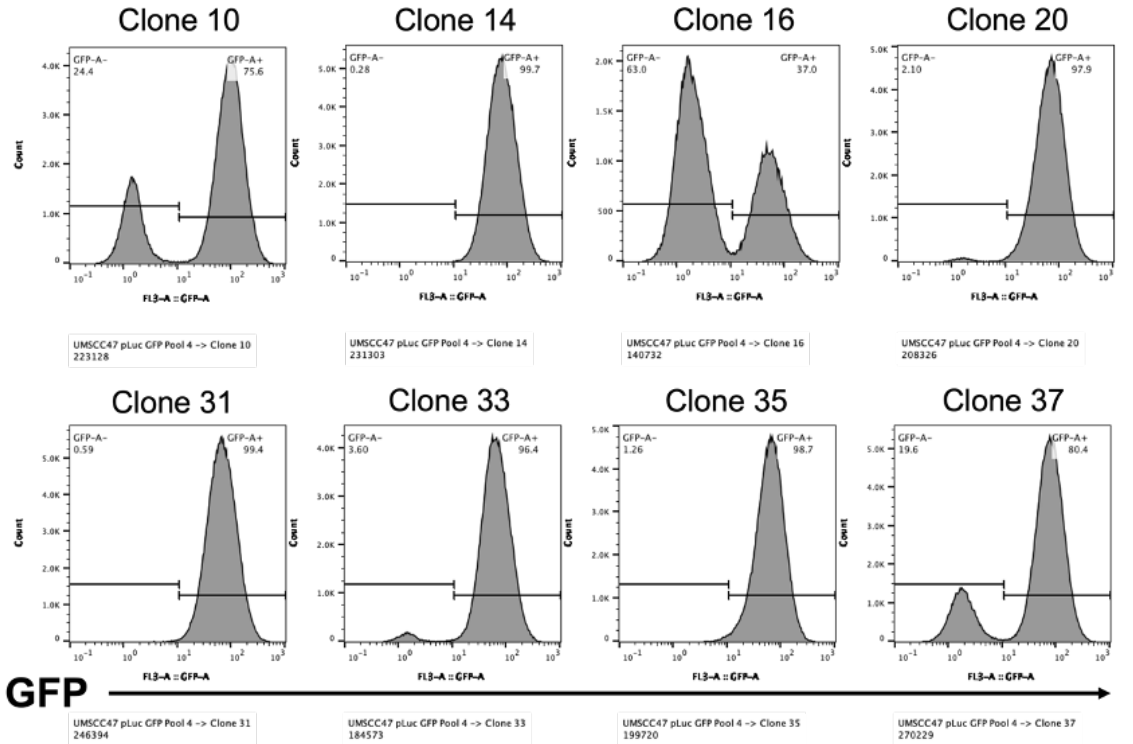
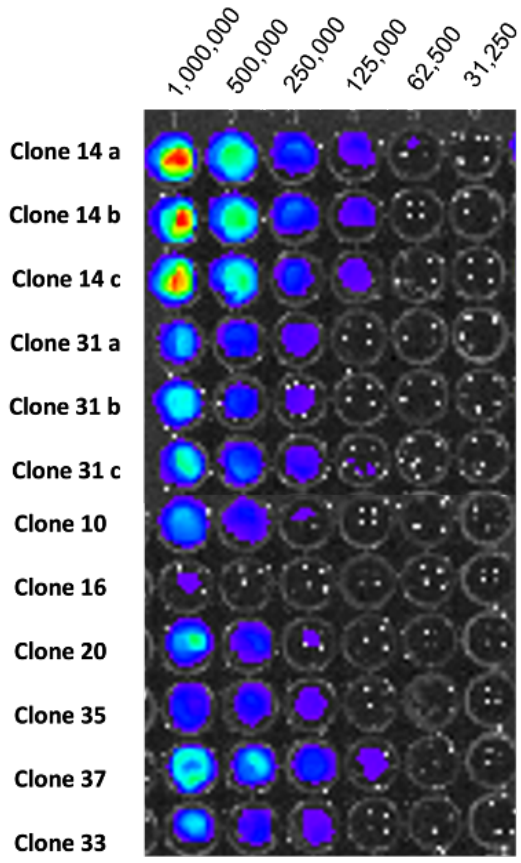


Figure 13 – Secondary screening of 8 stable clones. GFP expression by flow cytometry represented as **(A)** a scatter plot and as **(B)** a histogram depicting the percentages of GFP$^+$ labeled cells.

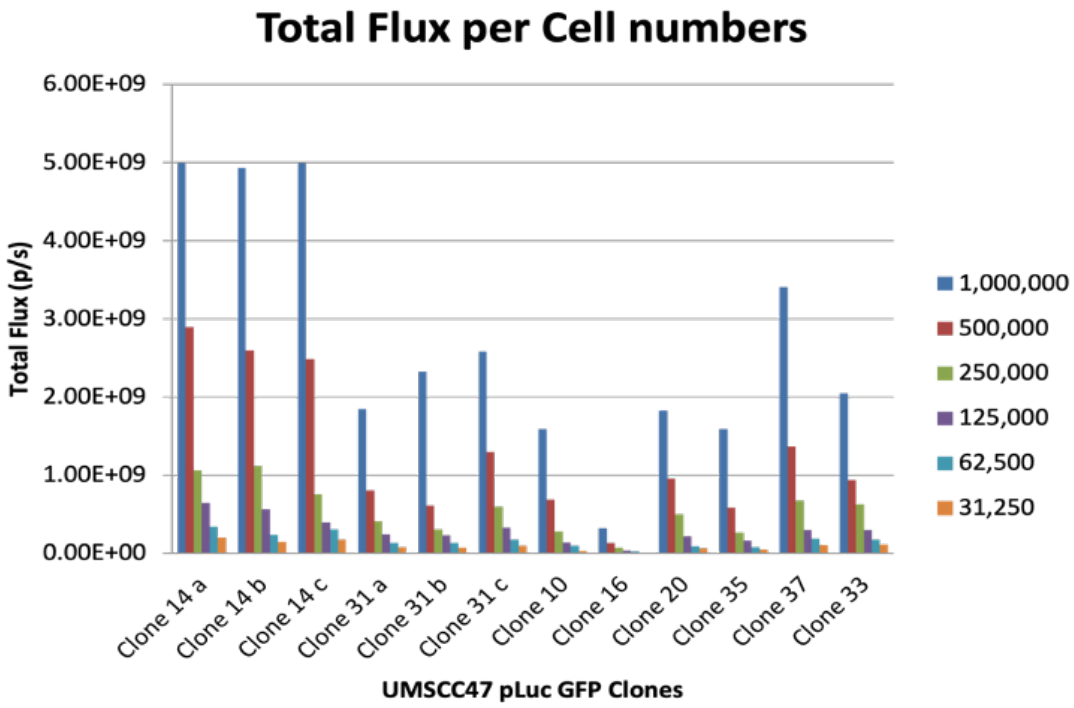
Clone #	GFP % (n)
14	98.96 (5)
31	98.75 (8)
35	98.29 (7)
20	97.04 (4)
33	95.37 (6)
37	80.52 (6)
10	70.40 (6)
16	57.26 (7)

Table 3 – Secondary screening of GFP expression in 8 stable clones. Average percentages of GFP^+ population as measured by flow cytometry. (n = number of repeats)

A)



B)



C)

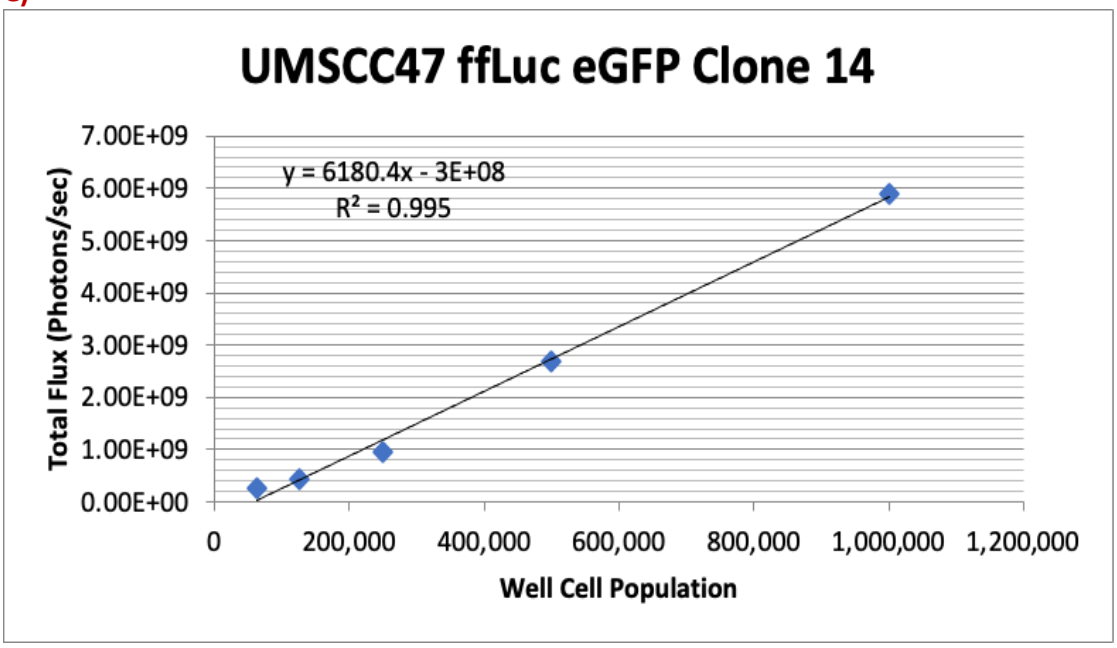


Figure 14 – (A) Representative image of bioluminescence detection of luciferase labeled clones at the indicated concentrations. **(B)** Graph of quantitative bioluminescent signal (photons/sec) of UM-SCC47 *ffLuc*-eGFP clones in each well. **(C)** Plot of linear relationship between the cell number and bioluminescent intensity detected, and linear regression calculated for the line of best fit.

Dose-Finding Study:

Before proceeding to the efficacy experiment, we optimized the concentration of GA-OH *in vivo* to a tolerable dose in mice. Our initial GA-OH dose was selected based on the literature search of a parent analog compound, gambogic acid (GA), which yielded 5 mg/kg as relatively tolerable in rodents (Jia, Li, Hu, Zhu, & Chen, 2015; Qi et al., 2008). However, when we tested this concentration with our lead molecule, GA-OH, we observed some toxicity. The day following the first injection, the animals presented with swelling, redness, and tenderness at the injection site **(Figure 15a).** We therefore tested two lower dosages of GA-OH: 3 mg/kg and 1 mg/kg. The GA-OH concentration of 3 mg/kg still manifested reactions and minor irritations to the treatment **(Figure 15b)**. Hence, we focused next on dosages below 2 mg/kg with a goal of finding a dose more tolerable and manageable in mice **(Figure 15c)**. We were able to conclude that concentrations below 1 mg/kg were non-toxic to animals, therefore we proceeded with 0.6 mg/kg as a dose predicted to minimize toxicity and maximize efficacy **(Figure 15d).**

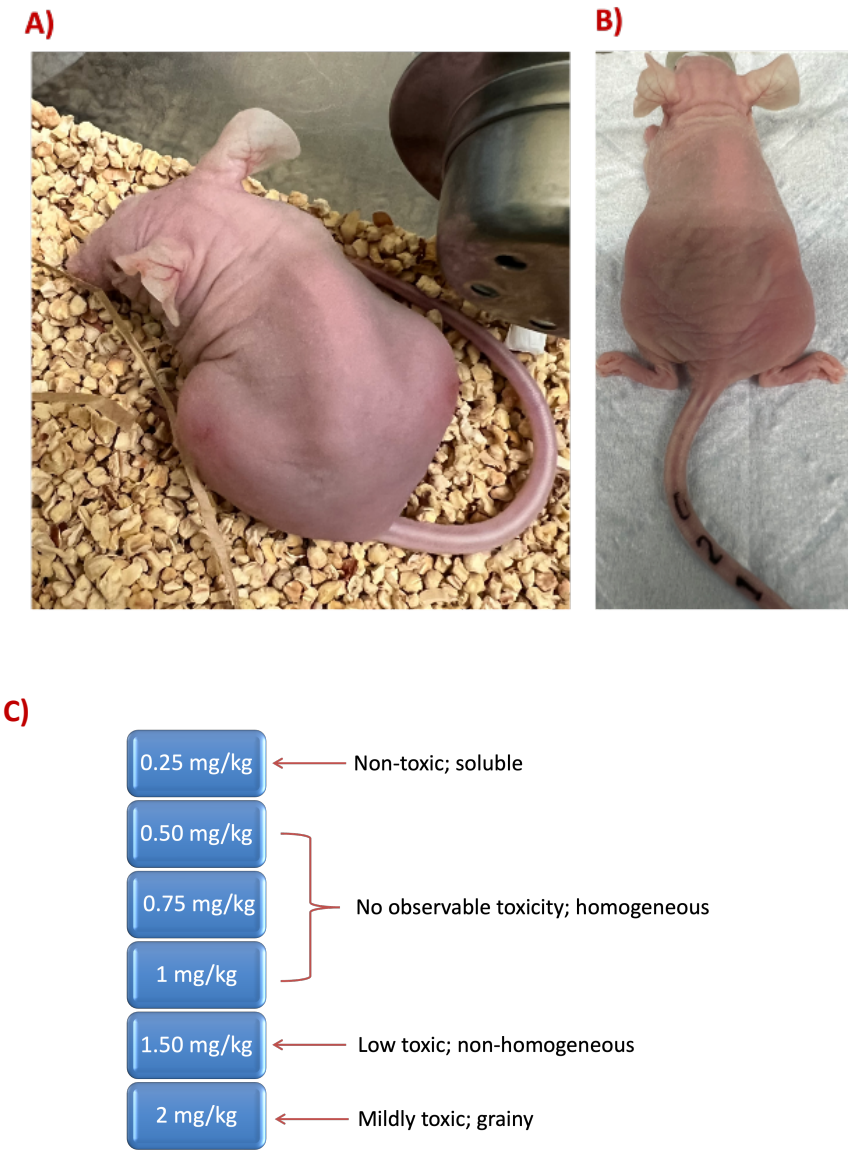
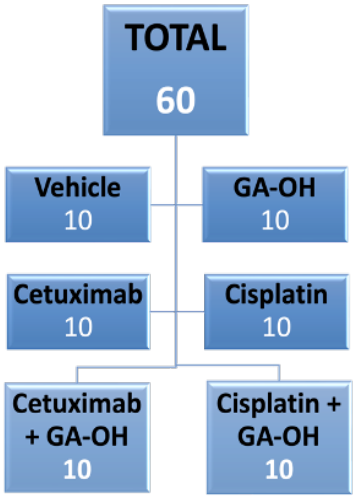


Figure 15 – GA-OH dose response study. Representative picture of mouse treated with 5 mg/kg **(A)** and 3 mg/kg **(B)** of GA-OH. Lower GA-OH dosage range tested **(C).** Selected GA-OH concentration for the antitumor efficacy study **(D).**

GA-OH Antitumor Efficacy Experiment

To determine efficacy, we injected the selected clone, UM-SCC47-*ffLuc*-eGFP Clone #14, in the mice to create xenografts and began the treatment regimen as outlined in Figure 3, a week after tumor injection. Groups consisted of: A) Vehicle B) GA-OH alone C) Cisplatin alone D) Cisplatin + GA-OH E) Cetuximab alone F) Cetuximab + GA-OH **(Figure 16a)**. On day 1, GA-OH or vehicle (saline) was administered; and on Day 2, an additional chemotherapeutic agent (10 mg/kg cetuximab or 5 mg/kg cisplatin) was administered intraperitoneally in addition to the small molecule. No injections were made on Day 3, and this 3-day cycle was repeated 5 times (for a total of about 2 ½ weeks) **(Figure 16b)**. At the end of the timeline, we sacrificed the remaining mice through CO_2 narcosis followed by exsanguination.

A)



A	Vehicle
B	GA-OH
C	Cisplatin
D	Cisplatin + GA-OH
E	Cetuximab
F	Cetuximab + GA-OH

B)

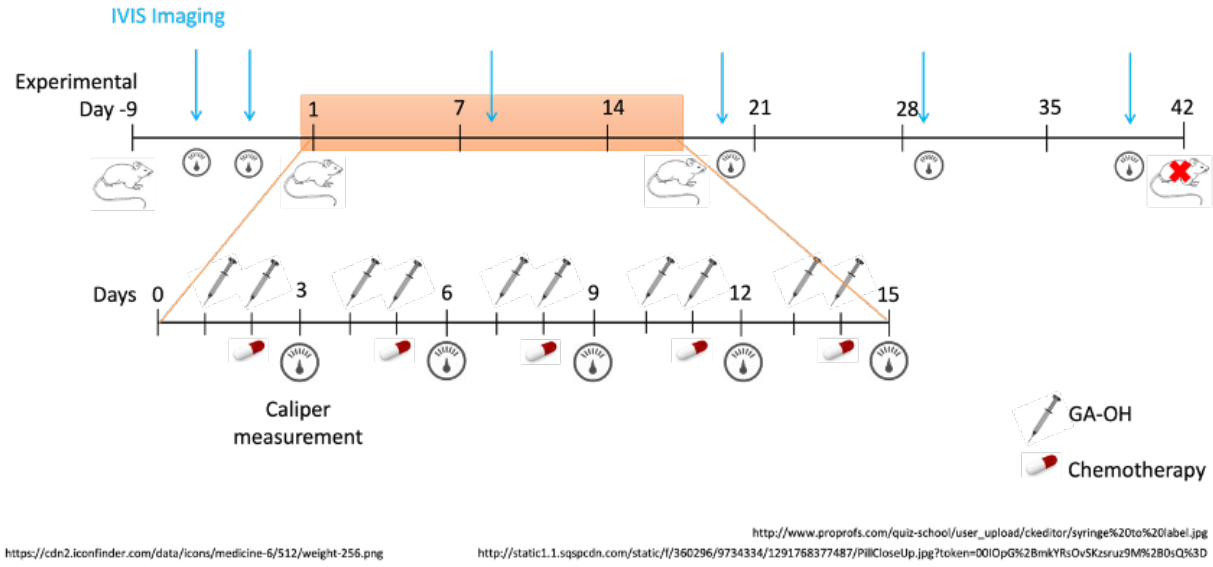


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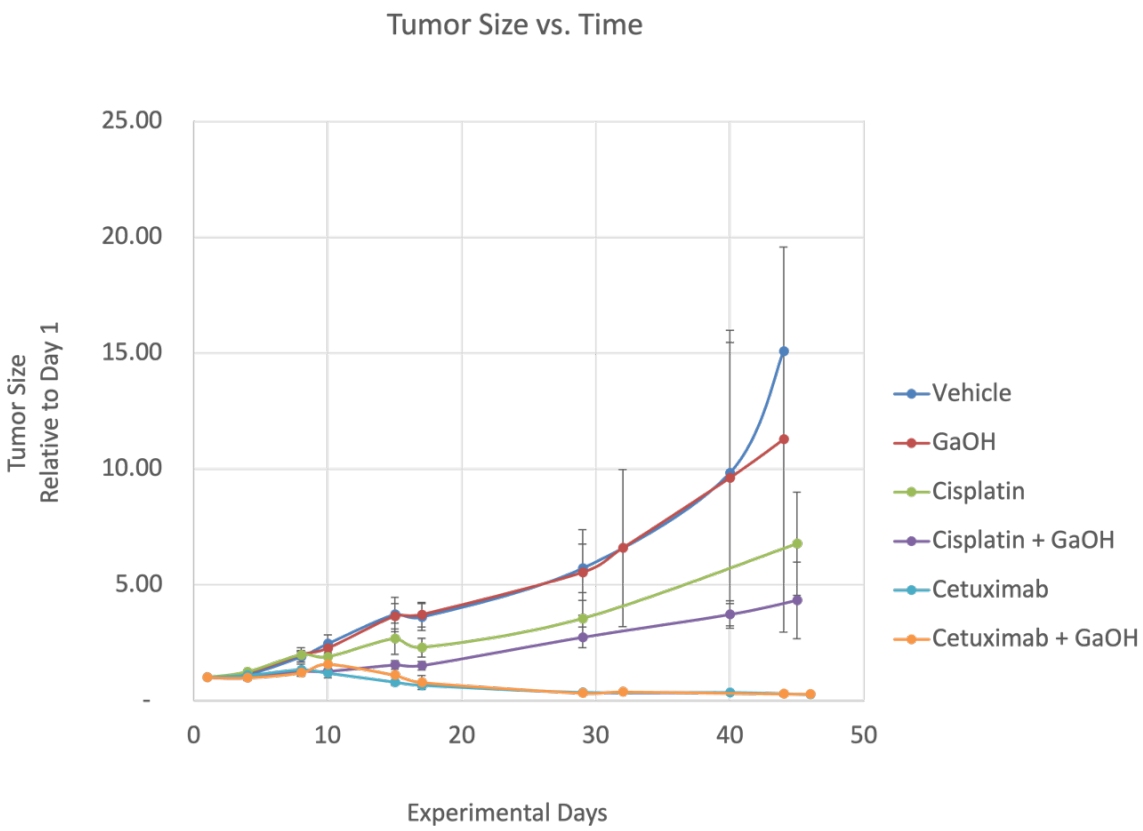
Figure 16 – (A) Group of treatments. **(B)** Scheme of treatment timeline.

Caliper Measurements:

Tumor growth was monitored by both caliper measurements and using the IVIS Lumina III. Based on our caliper measurements, we were able to conclude that as anticipated, tumors grew in a relatively linear manner in control animals, indicating a functional *in vivo* model. Additionally, both groups treated with cetuximab (single or in combination with GA-OH) not only slowed but regressed tumor growth. Moreover, we found that GA-OH alone did not slow HNSCC tumor growth. However, when GA-OH was paired with cisplatin, it enhanced the efficacy of cisplatin. These tumor patterns can be visualized quantitatively through the growth curves (**Figure 17a**). When we analyzed the addition of GA-OH in each of the chemotherapeutic regimens before and immediately after the treatment period, cisplatin was the only combination treatment where we observed a difference compared to its single treatment **(Figure 17b).** To determine the level of enhancement by the combination of GA-OH and cisplatin compared to cisplatin treatment alone, we further analyzed the curves through the Generalized Least Squares Regression (GLSR).

A)


Tumor Size vs. Time
Tumor Size
Relative to Day 1
25.00
20.00
15.00
10.00
5.00
-
0
10
20
30
40
50
Experimental Days
Vehicle
GaOH
Cisplatin
Cisplatin + GaOH
Cetuximab
Cetuximab + GaOH

B)

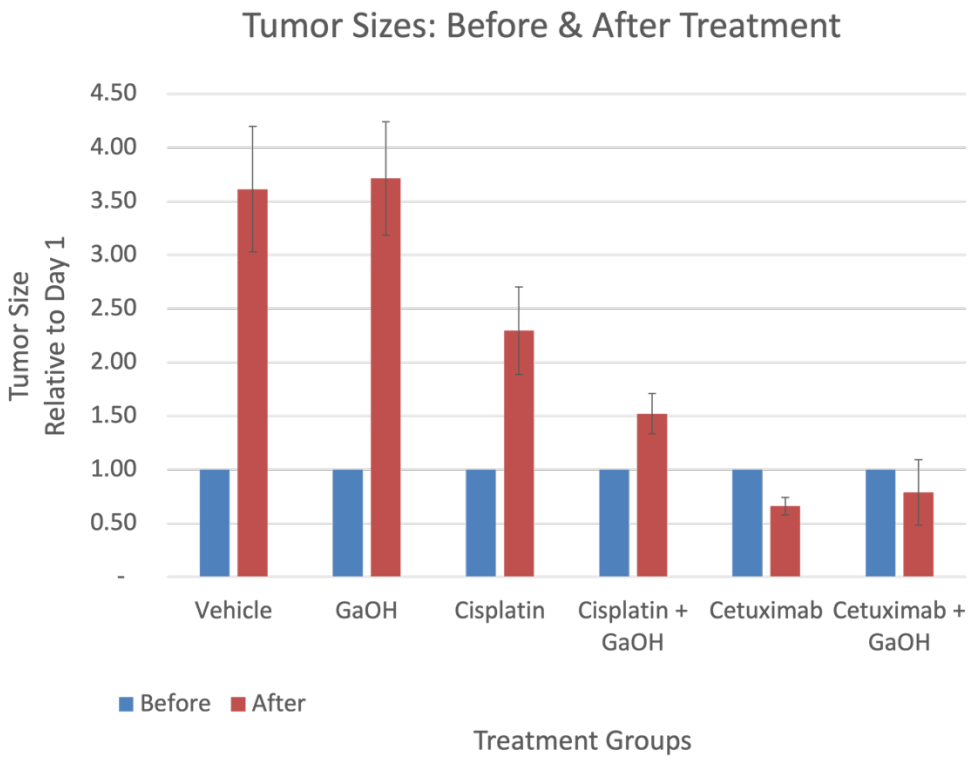


Figure 17 – Antitumor Efficacy Study. **(A)** Tumor growth curve. **(B)** Tumor measurements before (Day 1/Day 1) and immediately after the treatment regimen (Day 16/Day 1), as represented relative to tumor measurements before treatment.

Three group comparisons were performed: 1. Cisplatin + GA-OH compared to Vehicle, 2. Cisplatin + GA-OH compared to Cisplatin, and 3. Cisplatin + GA-OH compared to GA-OH. For data visualization purposes, tumor size linear regression for each group over six follow up time points are shown in **Figure 18** for each of the three group comparisons.

The effects of group assignment, time, and group assignment over time interaction for each comparison are displayed in **Table 1**. As expected, no significant differences in baseline tumor size were observed between groups in any comparison ([1] p = 0.0405, (Moore & Mehta) p = 0.0954, [3] p = 0.9251). In all groups, a significant increase in tumor size was observed over time ([1] p = 0.0000, (Moore & Mehta) p = 0.0000, [3] p = 0.0013). In all comparisons, tumor growth rate was significantly lower in group D (Cisplatin + GA-OH) ([1] p = 0.0009, (Moore & Mehta) p = 0.0001, [3] p = 0.0130).

Group Comparison 1: Cisplatin + GA-OH (D) versus Vehicle (A) ***(Figure 18a)***

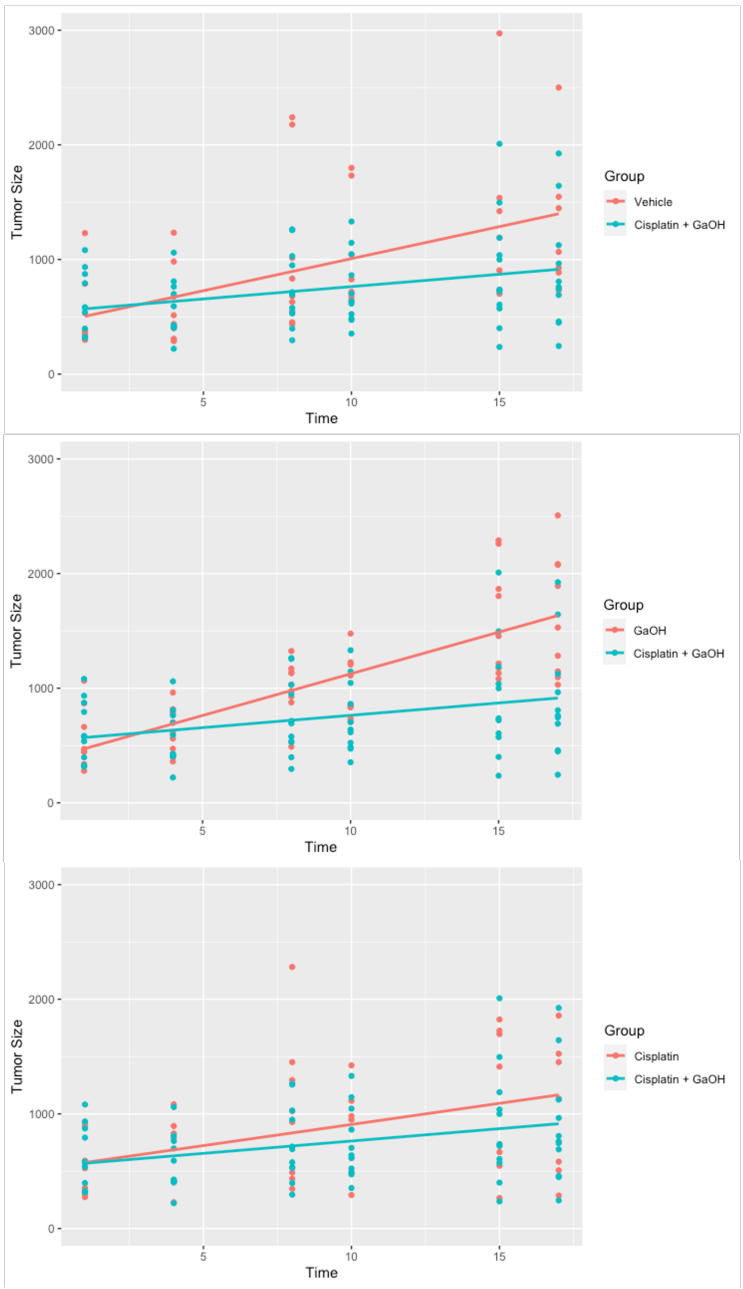
No significant differences in tumor size were observed between groups at treatment initiation (Day 1) (p = 0.0405). There was however a significant increase in Tumor Size over time in both groups (63.757 mm^3 per day, **** p = 0.0000). The Group x Time interaction was also significant, with the Cisplatin + GA-OH group displaying -16.179 mm^3 less tumor growth per day than the Vehicle group (*** p = 0.0009).

Group Comparison 2: Cisplatin + GA-OH (D) versus GA-OH (B) ***(Figure 18b)***

No significant differences in tumor size were observed between groups at treatment initiation (Day 1) (p = 0.0954). There was however a significant increase in tumor growth over time in both groups (95.201 mm^3 per day, **** p = 0.0000). The Group x Time interaction was also significant, with the Cisplatin + GA-OH group displaying -24.419 mm^3 less tumor growth per day than the GA-OH group (**** p = 0.0001).

Group Comparison 3: Cisplatin + GA-OH (D) versus Cisplatin (C) ***(Figure 18c)***

No significant differences in tumor size were observed between groups at treatment initiation (Day 1) (p = 0.9251). There was however a significant increase in tumor growth over time in both groups (115.781 mm^3 per day, ** p = 0.0013). The Group x Time interaction was also significant, with the Cisplatin + GA-OH group displaying -33.718 mm^3 less tumor growth per day than the Cisplatin group (* p = 0.013).



Effect	Value	p-value
Group	84.564	0.0405
Time	63.757	0.0000
Group : Time	-16.179	0.0009

Effect	Value	p-value
Group	104.296	0.0954
Time	95.201	0.0000
Group : Time	-24.419	0.0001

Effect	Value	p-value
Group	182.958	0.9251
Time	115.781	0.0013
Group : Time	-33.718	0.0130

Figure 18 – GSLR Statistical Analysis of treatment groups: **(A)** Cisplatin + GA-OH (D) vs. Vehicle (A) , **(B)** Cisplatin + GA-OH (D) vs. GA-OH (A), **(C)** Cisplatin + GA-OH (D) vs. Cisplatin (C). Generalized least squares regression was used to assess the effects of time, group, and group time interaction on tumor size. Coefficient parameters for each group comparison are displayed in Table 4.

Table 4: The Effects of Group, Time, and Group x Time Interaction on Tumor Size

Group Comparison 1: Cisplatin + GA-OH (D) versus Vehicle (A)

Effect	Value	p-value
Group	84.564	0.0405
Time	63.756	0.0000
Group x Time	-16.179	0.0009

Group Comparison 2: Cisplatin + GA-OH (D) versus GA-OH (B)

Effect	Value	p-value
Group	104.296	0.0954
Time	95.201	0.0000
Group x Time	-24.419	0.0001

Group Comparison 3: Cisplatin + GA-OH (D) versus Cisplatin (C)

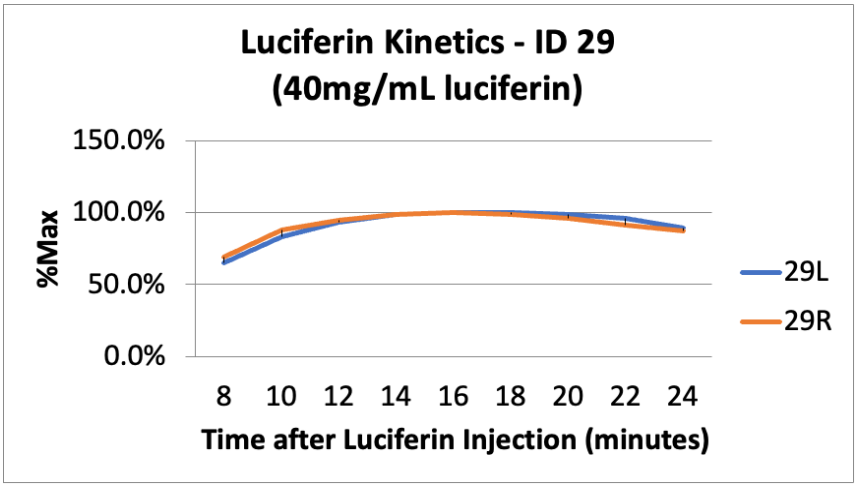
Effect	Value	p-value
Group	182.958	0.9251
Time	115.781	0.0013
Group x Time	-33.718	0.0130

Chemiluminescence Measurements:

For BLI measurements, sequence image acquisitions were started 13 minutes after luciferase substrate injection to ensure consistent photon flux, as determined from bioluminescence imaging kinetic studies in the same xenograft model. Based on the BLI kinetics in our xenograft model, *firefly luciferase* was shown to peak at around 16 minutes on average (**Figure 19a**). Therefore, for the tumor inhibition study, we timed our acquisition times to start at 13 minutes after luciferin injection and captured a sequence of 37 images, which lasted for about 6 min.

We optimized BLI measurements by adjusting the binning, exposure times, mode of substrate administration, luciferin concentrations, and acquisition times. However, we were unable to produce mathematically coherent tumor sizes that aligned with our caliper measurements. For this reason, tumor curves from BLI signal are unavailable, though we have provided BLI images of the growth of tumors in representative individual mice for visualization purposes (**Figure 19b**). Further optimizing BLI measuring and imaging tools remains a goal for future animal studies.

A)



B)

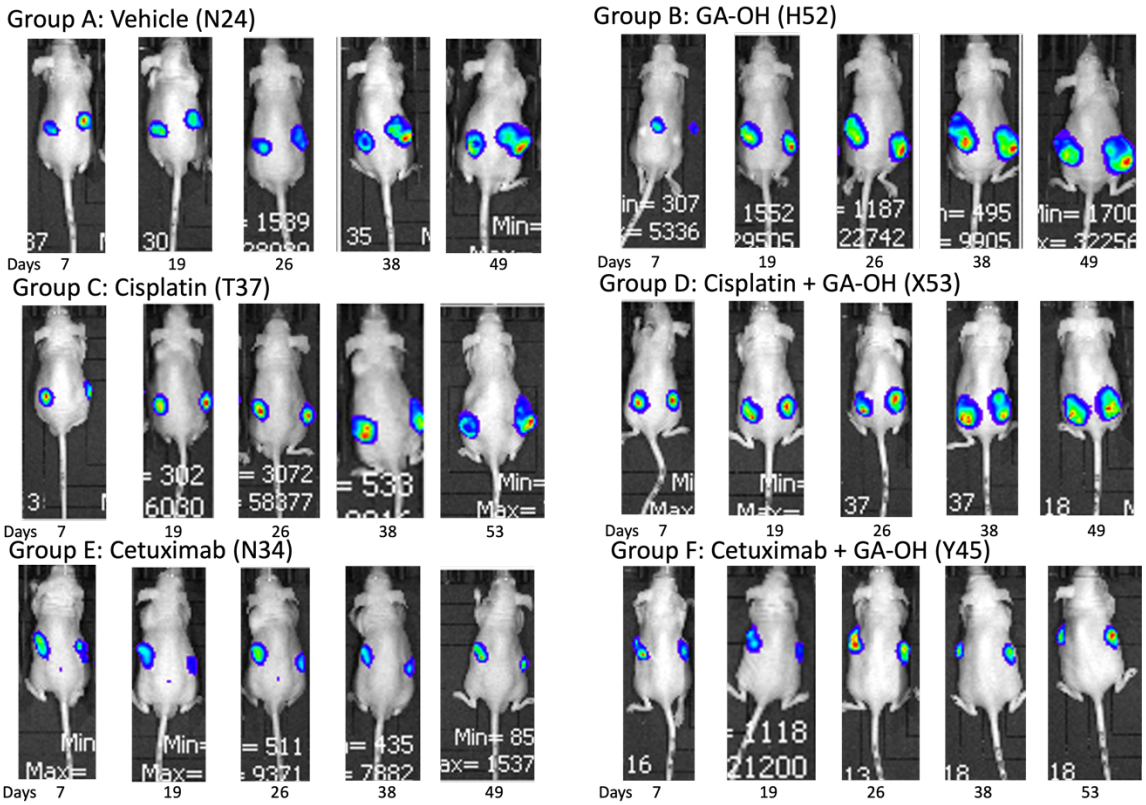


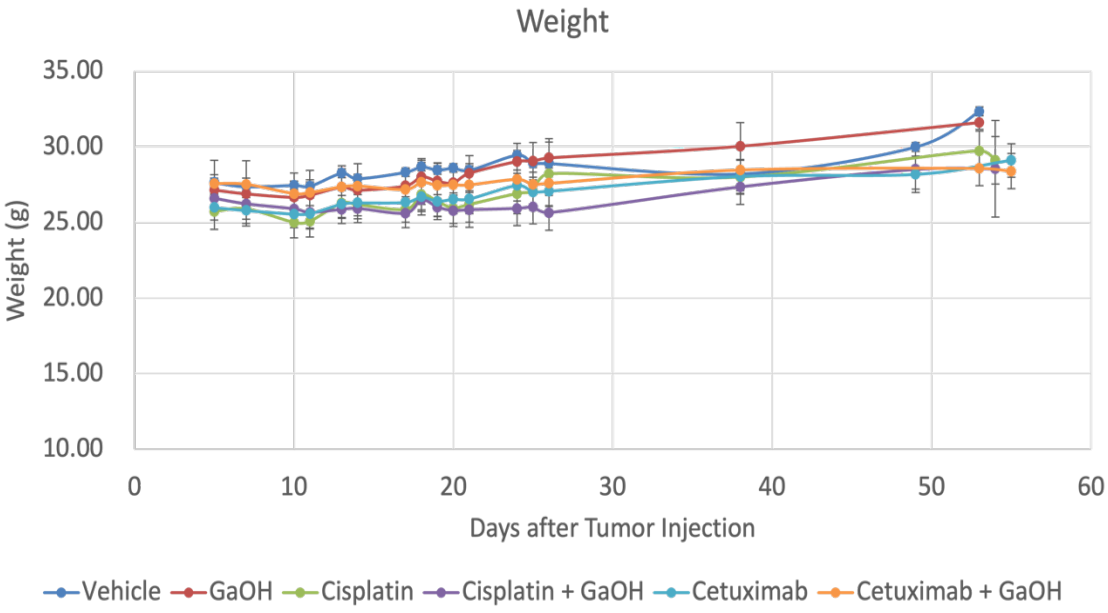
Figure 19 – (A) Representative BLI kinetic measurements of HPV⁺ UM-SCC47 tumors in a HNSCC xenograft model. **(B)** Representative mice BLI images of tumor growth over time for each treatment group (Mouse N24 represents Group A; mouse H52 represents Group B; mouse T37 represents Group C; mouse X53 represents Group D; mouse N34 represents Group E; mouse Y45 represents Group F)

GA-OH Toxicity Results:

We performed clinical observations for any changes indicative of declining health, and watched for signs of toxicity including hunched posture, lethargy or severe weight loss. Based on these assessments, physical or behavioral observations were deemed acceptable with no overt signs of toxicity noted. For example, a significant decrease in weight is indicative of toxicity, and we found that mice from each group had relatively minor variance in weight with no significant differences noted **(Figure 20a)**. Moreover, the evaluation of major internal organs (liver, kidneys, and spleen) post-mortem showed no toxicity and relatively consistent organ weight between the treatment groups **(Figure 20b)**. We noted that one mouse in the cetuximab treated group had an enlarged liver upon gross necropsy. Upon close observation, we noted that this same mouse also appeared to have very elevated levels of Alanine Transaminase (ALT), as indicated in **Figure 21a**.

We also performed hematological and clinical chemistry tests on blood collected immediately after euthanasia. The complete blood count panel had minor changes when comparing treatment regimens, but no significant disparity overall **(Table 5)**. The toxicity chemistry panel showed small variations between treatments **(Table 6)**, except when it came to aspartate aminotransferase (AST) (** $p = 0.0057$) and creatine kinase (CK) (**** $p < 0.0001$), which were significantly higher in the Cisplatin + GA-OH group (D) compared to vehicle (A) **(Figure 21a)**. Additionally, when compared with the single-treated cisplatin (C), the combined treatment (D) had an appreciable elevation of AST that did not reach statistical significance ($p = 0.1265$). However, creatine kinase continued to exhibit a highly significant increase in group D vs. group C (*** $p = 0.0005$) **(Figure 21b)**.

A)


Weight
35.00
30.00
25.00
20.00
15.00
10.00
Weight (g)
0
10
20
30
40
50
60
Days after Tumor Injection
Vehicle
GaOH
Cisplatin
Cisplatin + GaOH
Cetuximab
Cetuximab + GaOH

B)

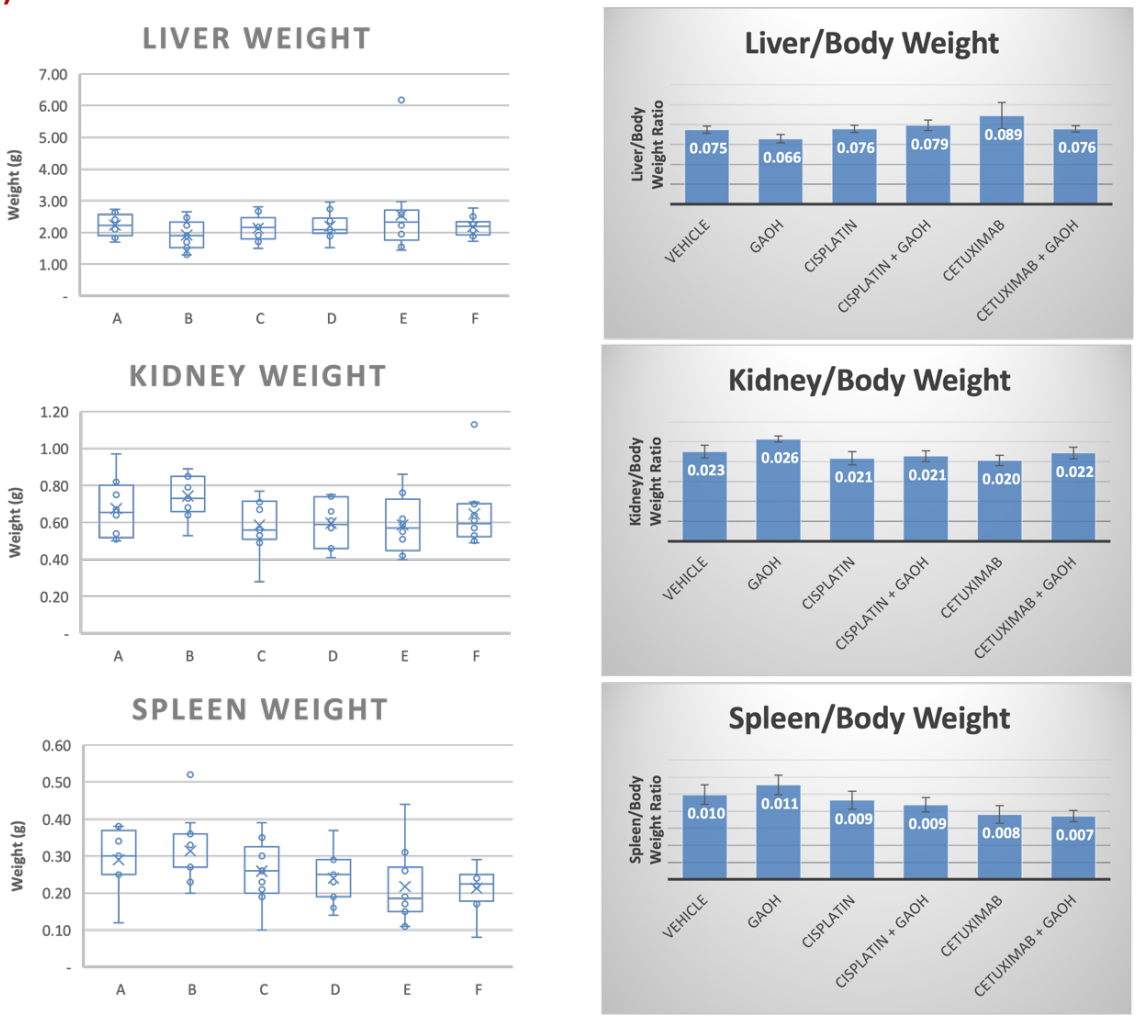


Figure 20 – GA-OH Toxicity Results. **(A)** Body weight for each treatment group. **(B)** Major internal organ weight for each treatment group and the organ/body weight ratio.

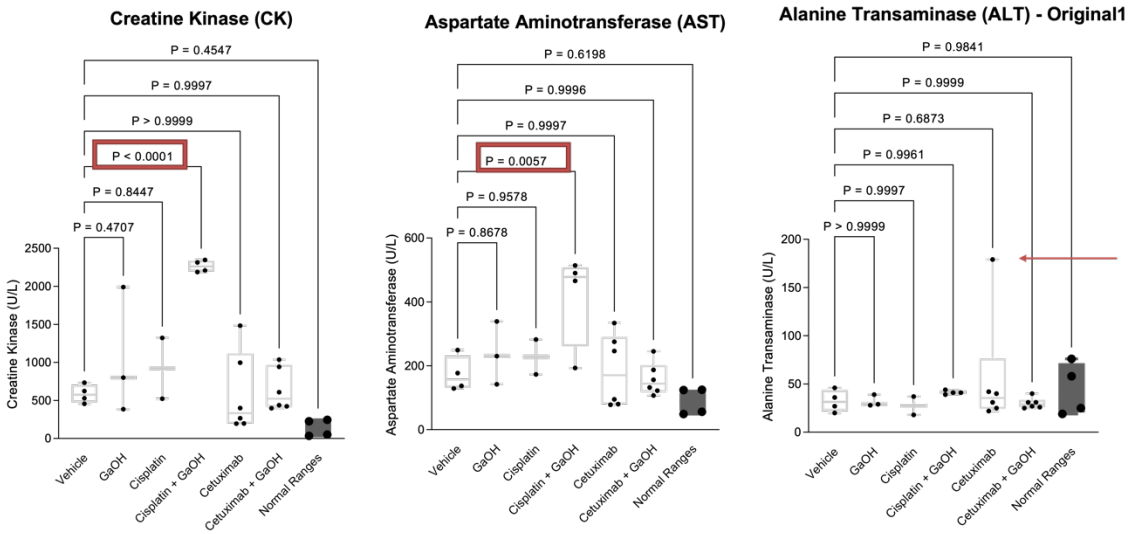
Blood Tests	Vehicle	GA-OH	Cisplatin	Cisplatin + GA-OH	Cetuximab	Cetuximab + GA-OH	Normal Range
Reticulocyte (%)	4.33	3.67	4.60	4.10	4.05	3.58	
Absolute Reticulocyte (%)	427.25	369.33	407.00	370.50	416.50	384.40	
RBC (M/uL)	9.87	10.08	8.86	9.02	10.34	10.67	7.87-12.00
Reticulocyte Hemoglobin Content (pg)	18.18	18.03	18.05	18.50	18.67	18.42	
HGB (g/dL)	15.03	15.00	13.80	14.18	15.58	16.02	11.90-17.90
Hematocrit (%)	49.55	49.27	46.60	47.45	52.30	54.24	35-62.1
MCV (fL)	50.50	49.00	52.50	52.50	50.83	51.00	41.8-62.4
MCH (pg)	15.25	14.90	15.60	15.70	15.10	14.98	13.4-17.8
MCHC (g/dL)	30.35	30.43	29.60	29.90	29.83	29.52	23.3-38.5
Platelet Count (K/uL)	805.00	906.33	819.00	667.50	395.67	458.20	784-1812
Neutrophil (%)	45.50	44.67	49.00	49.28	26.20	28.84	15.93-64.57
Neutrophil (/uL)	2520.00	1571.33	4437.00	2411.75	2134.67	1669.20	820-6060
WBC (K/uL)	6.00	3.57	8.50	5.08	8.37	5.98	1.94-12.59
Lymphocyte (/uL)	2348.50	1594.67	3587.00	2304.00	4987.33	3629.40	960-8200
Lymphocyte (%)	39.25	44.00	45.00	43.53	60.27	59.18	31.34-79.22
Monocyte (/uL)	410.25	319.33	297.50	319.25	983.83	455.40	90-630
Monocyte (%)	6.50	8.67	4.50	6.25	10.25	7.92	0.56-7.04
Eosinophil (/uL)	140.00	71.67	119.00	33.25	253.33	215.80	0-790
Eosinophil (%)	2.50	2.33	1.00	0.83	3.17	3.88	0.21-5.52
Basophil (/uL)	0.00	9.67	0.00	7.00	7.50	10.00	0-90
Basophil (%)	0.00	0.33	0.00	0.13	0.12	0.18	0-1.73

Table 5 – Hematological Test: Complete Blood Count panel

Blood Tests	Vehicle	GA-OH	Cisplatin	Cisplatin + GA-OH	Cetuximab	Cetuximab + GA-OH	Normal Range
ALP (U/L)	32.25	43.67	40.50	52.75	73.17	62.17	34-134
AST (U/L)	173.00	237.00	227.50	**415.75**	184.67	158.17	49-125
ALT (U/L)	32.25	32.00	27.50	41.25	**56.50**	30.00	19-76
Creatine Kinase (U/L)	585.25	1058.33	921.50	**2264.25**	589.67	640.83	35-246
Albumin (g/dL)	2.85	2.77	2.70	2.90	2.88	3.00	2.9-4.0
Total Bilirubin (mg/dL)	0.23	0.23	0.15	0.20	0.22	0.20	0.16-0.5
Total Protein (g/dL)	5.00	5.27	5.05	5.28	4.88	5.10	4.6-6.0
Globulin (g/dL)	2.15	2.50	2.35	2.38	2.00	2.10	1.5-2.3
BUN (mg/dL)	24.00	25.00	25.00	32.50	28.00	27.17	15-33
Creatinine (mg/dL)	0.18	0.10	0.15	0.18	0.15	0.18	0.1-0.5
BUN/Creatinine Ratio	164.18	250.00	195.00	112.50	89.45	158.33	

Table 6 – Serum Chemistry Toxicity Panel of each treatment group

A)



B)

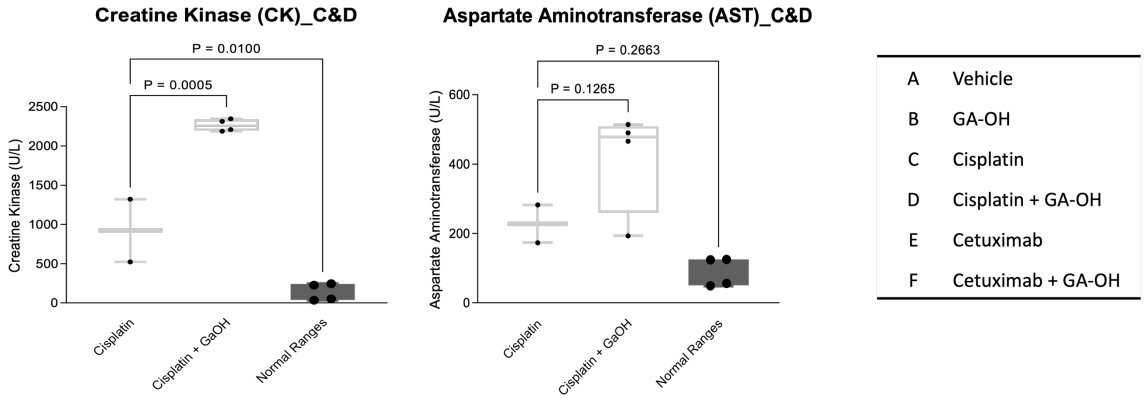


Figure 21 – Chemistry panel. **(A)** Levels of Creatine Kinase (CK), Aspartate Aminotransferase (AST), and Alanine Transaminase (ALT) for each treatment group. **(B)** Levels of CK and AST between groups C vs D.

Discussion

The targeted therapy cetuximab is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), and HNSCC is frequently known to have an overexpression of EGFR (Ayuso et al., 2019; Luedke et al., 2012; Solomon, Young, & Rischin, 2018). It is particularly used for metastasis and recurring HNSCC. Cetuximab acceptable dosages in rodents ranged between 4-40 mg/kg, according to previous studies (Steiner et al., 2007). Cetuximab is an EGFR antagonist, and studies have shown that UM-SCC47 cells express EGFR (Husain et al., 2012; Khanal et al., 2018). Hence, our study has demonstrated very high efficacy with cetuximab in our HPV^+ HNSCC xenograft model established through UM-SCC47 tumor injections, consistent with findings reported in similar studies of xenografts from different disease models (Kwon et al., 2014). Interestingly, there is conflicting clinical evidence of whether cetuximab is effective in either HPV^+ or HPV^- patients (Mirghani & Blanchard, 2018). Cetuximab was widely used in the 2000's, however cisplatin has been shown to have better response in terms of patient outcome and survival and remains the first line standard of care (Krishnamurthy et al., 2022). In optimizing the concentrations of cisplatin, we found in the literature that it was tolerable to mice between the ranges of 1-10 mg/kg (Johnsson & Wennerberg, 1999). Because we wanted to test the efficacy of combination treatments, we selected dosages in the mid- and lower ranges to observe the effect of any combined treatments. Moreover, by using a lower dose of cisplatin, we were anticipating that the side effects of the cytotoxic agent on patients; i.e. vomiting, diarrhea, dry mouth, and hair loss; would be minimized.

We found cetuximab to be very efficacious in regressing and eliminating tumor growth, to the extent that we were unable to delineate GA-OH's effect in the combination treatment when compared to cetuximab treatment alone. In the case that we may use cetuximab in the future, we would consider a lower dosage to discern an amplification effect when combined with lead molecules. Based on the tumor growth curve, GA-OH alone was shown to have little to no effect on tumor growth, indicating low toxicity and low efficacy by itself, as expected. As expected, we also found that cisplatin significantly slowed tumor growth. Furthermore, when GA-OH was administered with cisplatin, the combination significantly amplified the effect of cisplatin *in vivo* (* $p = 0.013$), confirming our initial prediction. Furthermore, we were able to demonstrate that our tumor growth model was functional, and the xenograft model established in our lab is apt for future small molecule testing. Previous bench experiments in our lab have shown that small molecule inhibitors of E6 could sensitize HPV^+ cells in combination with chemotherapy *in vitro*, and the animal study described here has provided proof of concept that inhibiting E6 can reduce tumor growth in combination with chemotherapy *in vivo*. This has strengthened the evidence that a combination of a small E6 inhibitor with chemotherapy may be a feasible therapeutic approach.

Regarding the toxicity of GA-OH, concentrations above 2mg/kg manifested visible reactions of redness of skin, inflammation, tenderness, and swelling of the skin at the site of injection in the dose-response study. These reactions to GA-OH were evident within 24 hours and most apparent at 5mg/kg dosages **(Figure 15a)**. After a series of lower dosage testing between 0.25 mg/kg and 2 mg/kg, we narrowed down the higher end of doses

likely to be nontoxic between 0.5 and 0.75 mg/kg **(Figure 15c)**. The 0.75 mg/kg concentration accounted for some minor signs of toxicity in the form of tiny tumor ulcerations and cavitations, while no visible signs were evident in dosages of 0.5 mg/kg GA-OH. In search for the highest dose that would fail to demonstrate toxicity, we selected 0.6 mg/kg of GA-OH for our tumor inhibition study.

The toxicity of each treatment group was further evaluated during the antitumor efficacy study. Physical and behavioral assessments were found to be mostly normal. All full body weights as well as the weight of major internal organs appeared to be within the acceptable parameters, with the exception of one mouse in the cetuximab treated group which was observed post-mortem to have hepatomegaly. Upon delving into the serum chemistry test of this mouse, the toxicity results were mostly within range except for a very elevated level of ALT (179U/L, [normal ranges 19-58U/L]), indicating severe liver injury. Liver damage can be caused by liver infection or drug toxicity, among other causes. Cetuximab is shown to cause hepatic enzyme elevation but no clinical liver injury. Since only one mouse in the treatment group exhibited an enlarged liver and elevated levels of ALT, there is not enough evidence to know whether any liver injury was due to the cetuximab treatment.

Blood chemistry comparison and analysis between the treatment groups demonstrated higher levels of CK (2264 U/L, **** $p < 0.0001$) and AST (415 U/L, ** $p = 0.0057$) in the combined cisplatin and GA-OH treated group as compared to vehicle **(Figure 21a)**. AST is another liver enzyme that may indicate liver damage at very elevated levels. When we compared the levels of the combined group to the cisplatin single treated

group, CK continued to be significantly higher (*** p = 0.0005) **(Figure 21b)**. Creatine kinase is an enzyme prevalent in skeletal muscle that plays a key role in high energy phosphate metabolism (Roman, Wieringa, & Koretsky, 1997), and it is a biomarker of muscle damage or intense exercise (Kim & Wierzbicki, 2021). In extreme cases (>5000IU/L), rhabdomyolysis, a rare muscle disease where the muscles break down, followed by acute kidney injury is diagnosed. Elevated CK levels can also emerge from drug interactions that are metabolized through the cytochrome P450 3A4 pathway (Kim & Wierzbicki, 2021). Lipid lowering drugs such as statins are commonly known to raise the levels of CK in patients. Other drugs that might elevate CK are antiretrovirals and oncologic drugs such as BRAF inhibitors and PD-L1 inhibitors (Kim & Wierzbicki, 2021). Interestingly, cisplatin has also been shown to be metabolized or cleared systemically through cytochrome P450 (CYP) CYP3A and to increase muscle wasting (Conte et al., 2020; Goldstein et al., 2013). Additionally, GA, the parent analogue of GA-OH, is known to be a potent inhibitor of CYP3A4 (W. Liu et al., 2015), affecting drug interactions by concomitant drug clearance and metabolism reduction, and thus enhancing the drug's toxicity as well as its therapeutic effect (Conte et al., 2020; Scripture, Sparreboom, & Figg, 2005). GA has been widely studied and researched over the last couple of decades as a cancer therapeutic option, more generally in breast, lung and liver cancer (Hatami, Jaggi, Chauhan, & Yallapu, 2020). In addition, preclinical and clinical evidence have indicated that GA has antitumor effects on several cancers, including breast cancer, lung cancer, prostate cancer, ovarian cancer, glioma, melanoma, head and neck cancer and cervical cancer (Banik et al., 2018). Furthermore, GA has demonstrated to have chemo

sensitization effects on several cancer types, and thus garnered interest as an anti-cancer agent in combinatorial therapies (Banik et al., 2018). Additionally, Wang *et al*. has shown that GA specifically increases cisplatin's chemotherapeutic effect in non-small cell lung cancer (Wang et al., 2014). Therefore, we speculate that when GA-OH, a derivative of the P450 inhibitor gambogic acid, is combined with the cytotoxic agent cisplatin, clearance of cisplatin is reduced, leading to an elevated plasma concentration of cisplatin in the body. Hence, cisplatin's chemotherapeutic effect as well as its toxicity became evidently more pronounced in our study, increasing its antitumor efficacy as well as its muscle wasting toxicity, indicated through CK's significant elevation. This finding warrants further analysis with a larger sample to confirm the results. We will keep this toxicity marker in mind as we perform any future experiments pairing GA-OH and cisplatin together. In conclusion, behavioral and physical assessment, body weight, organ necropsy, and blood tests, with the possible exception for CK, all pointed towards lack of significance toxicity and predicting tolerance.

Future studies combining different HNSCC treatment therapies in the mouse xenograft would corroborate our hypothesis. Our lab has shown that combined GA-OH treatment enhances radiotherapy efficacy *in vitro* (Chitsike et al., 2023). Consequently, translating this study into an *in vivo* context would further validate our working model. We have also established a cervical cancer model that can test small compounds in an HPV^{+} cervical cancer xenograft. Evaluating the small compound's efficacy in this model could demonstrate GA-OH's versatility in combating other HPV malignancies *in vivo*. This small molecular therapy approach has also been shown to display low toxicity at low

concentrations, allowing for reduced concentrations of cytotoxic agents that could potentially lower side effects, all while increasing the effectiveness of chemotherapy. Additionally, GA-OH would further need to be developed to improve potency and drug-likeness for clinical purposes. The limited number of effective therapies for HNSCC provides impetus for developing an effective, safe and targeted therapy for HPV patients. For these compelling reasons, efforts to continue to optimize the safety and effectiveness of GA-OH and other small molecule E6 inhibitors remain prioritized. Such continued endeavors to develop innovative and effective therapeutic approaches to combat HPV infection and its associated malignancies will considerably impact not only therapeutical but functional outcomes in this subset of HPV patients.

CHAPTER FOUR

OVERVIEW & FUTURE DIRECTIONS

Overview

The objective of this study was to test E6 small molecule inhibitors in an *in vivo*, HPV+ HNSCC tumor xenograft model that we will establish. Our hypothesis was that small molecule E6 inhibitors would increase the effectiveness of HPV^+ HNSCC therapy. First, we developed a system in which we could effectively test small molecules *in vivo*. We accomplished this by expanding our cancer xenograft model to incorporate an HPV^+ HNSCC model in addition to our existing cervical cancer model. Moreover, we enhanced the animal model to facilitate engraftment and produce more consistent tumor growth by using Matrigel. We also applied state-of-the-art luciferase reporter technology to visualize the tumors in real time *in vivo*.

Our lab has previously identified caspase 8 as a target of the HPV E6 oncoprotein (Tungteakkhun et al., 2010), joining previously identified molecules such as p53 on the list of molecules and pathways affected by HPV. Our lab also established a high-throughput screen assay using AlphaScreen technology to assess the interactions of small molecules in the context of these binding events. A query of a 2,000-compound library (ActiProbe 2K, from TimTec, LLC) , identified spinacine as a lead compound. After significant *in vitro* assessments and cell culture testing (Yuan et al., 2016), we made the transition to test the small molecule inhibitor in an animal model. In this study, as reported here, spinacine was not toxic to mice at doses up to and including 20 mg/kg. In this first *in vivo* tumor

inhibition study, however, spinacine did not yield evidence of significant efficacy either alone or when combined with apoptotic agents *in vivo (Chapter two)*.

Subsequently, our lab continued screening small molecule libraries to identify other E6 inhibitors. The 5K compound library from Kansas University yielded a new compound, gambogic acid. Further analyses led to an analog that was more effective, GA-OH (30-hydroxygambogic acid). GA-OH displayed higher specificity and effectiveness than our previous lead molecules *in vitro*, making it a more promising candidate. After Dr. Chitsike provided proof of principle that GA-OH was able to upregulate apoptotic mediators and sensitize HPV-infected cells *in vitro (Chitsike et al., 2021),* we proceeded to test this small molecule in an animal model.

Our animal studies included a dose-finding experiment that demonstrated GA-OH doses above 2 mg/kg to be toxic to mice but found the compound to be tolerable in doses below that. Furthermore, the antitumor efficacy study demonstrated that GA-OH alone did not reduce tumor growth; however, in combination with chemotherapy, GA-OH could significantly enhance the effectiveness of cisplatin *(Chapter three)*. This combined treatment revealed that most measured parameters did not change from control levels, though a couple of blood chemistry results demonstrated elevated levels of aspartate aminotransferase (AST) and creatine kinase (CK) compared to other treatment groups. Compared to the cisplatin treated group, the combined treatment group displayed an appreciable increase only in AST, while CK continued to be significantly elevated, warranting a larger sample of GA-OH and cisplatin paired treatments to confirm (or fail to confirm) these findings. No other toxicological assessments or parameters indicated a

decline in health in rodents. In this study, we were able to characterize GA-OH *in vivo*, including its optimal dosage, toxicity endpoints and tumor efficacy. We were also able to optimize other protocol parameters, such as dosages of the chemotherapeutic agents in these xenograft models, and the maximal BLI of luciferase-labeled tumors through kinetics measurements. In addition, we expanded the basement membrane matrix to include Cultrex as a suitable agent for xenograft tumor growth. This study has established the conceptual framework that small molecule E6 inhibitors can reduce HPV^{+} tumor growth *in vivo* when paired with chemotherapy. Further development of the effectual translation from the bench to the animal model will provide more insights of GA-OH activity and potentially create more opportunities to advance this compound towards the clinical setting.

Review Update

Since our publication of a review of therapeutic treatments and screening strategies (*Chapter one*), several notable advancements have been made in the field. The number of clinical trials for de-intensification of specifically HPV HNSCC has significantly increased over the last several years, as have the number of novel and targeted therapies developed and tested for HPV HNSCC. Currently, radiotherapy and chemotherapy remain the gold standard for patients with locally advanced stages or recurrent and metastatic disease (Sun et al., 2021), and radiotherapy or surgery single treatments are encouraged at early stages of disease progression based on NCCN. Standard of care for recurring cancer mainly includes chemotherapy, immunotherapy with PD-1-inhibitors, and a

targeted monoclonal therapy, cetuximab, all as either monotherapies or as a combination of these options (Ghosh et al., 2022).

Several papers have demonstrated favorable outcomes for minimally invasive surgical techniques, specifically transoral robotic surgery (TORS), indicating an overall survival of 80% in HPV patients (Pinkiewicz, Dorobisz, & Zatonski, 2022). Moreover, de-intensification radiotherapy clinical trials such as ECOG 3311, OPTIMA, and Checkrad-CD8 have resulted in positive outcomes in lowering dosages for HPV-associated patients (Sun et al., 2021; X. Zhou & Wang, 2022), and studies testing intensity modulated radiotherapy (IMRT) has also corroborated the excellent outcomes in this subset of patients (Zorzi et al., 2022). Cisplatin remains the first line standard of care for the treatment of the HPV subset of HNSCC patients after demonstrating higher efficacy than cetuximab in several clinical studies (Gillison et al., 2019). Multiple trials testing the efficacy of cisplatin in combination with other chemotherapeutic drugs or modalities are currently underway, such as EXTREME and ECOG 1308 (Ghosh et al., 2022). Due to cisplatin's cytotoxicity and deleterious side effects, the identification of other agents with less toxicity remains an important goal.

In terms of vaccines, the 2020 approval of the nonavalent prophylactic vaccine, Gardasil 9, for the prevention of several HPV cancers including HNSCC, has been a major step in the prevention of HPV-induced HNSCC, with a vaccine efficacy of 88-93.3%, and a population-level effect of 17% due to low uptake (Diana & Corica, 2021; Liao et al., 2022; J. Z. Zhou et al., 2021). Therapeutically, vaccines have demonstrated limited efficacy as a monotherapy. However, the combination of therapeutic vaccines with other modalities

has emerged to suggest higher effectiveness (Yan, Cowell, Tomkies, & Day, 2023). A few vaccines, such as ADXS-HPV and UCPVax, are now in clinical trials (Sun et al., 2021; Yan et al., 2023)

Several novel therapies have emerged in the areas of targeted therapies and immunotherapies. Targeted therapies that inhibit the PI3K/AKT/mTOR pathway, EGFR signaling, VEGF signaling, FGFR signaling, MEK/ERK signaling, MET signaling, CDK4/6 signaling, and Notch signaling, to name a few, have been developed (Li et al., 2023). A number of these inhibitors such as those that target PI3K/AKT/mTOR, have made it to clinical trials in Phase I/II (Ghosh et al., 2022).

Immune checkpoint inhibitors (ICI) have created a therapeutic niche following the approval of PD-1 inhibitors nivolumab (2016) and pembrolizumab (2019) as a frontline treatment for HNSCC for both HPV positive and negative HNSCC. Such results have shifted research toward immunotherapy in clinical studies (Ghosh et al., 2022; X. Zhou & Wang, 2022). ICI play a key role in interfering with the interactions between inhibitory receptors and their ligands, including programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and others (Damasio, Nascimento, Andrade, de Oliveira, & Calzavara-Silva, 2022; X. Zhou & Wang, 2022). Programmed death-1 (PD-1), a member of the CD28 receptor family, is expressed on activated immune cells such as T-cells, B-cells, monocytes, and dendritic cells (Damasio et al., 2022; Ghosh et al., 2022). When the ligand PD-1L binds and activates PD-1, it impairs and exhausts the immune system, thereby inhibiting anticancer responses of tumor-infiltrating lymphocytes and allowing tumor cells to evade immune surveillance. A myriad

of clinical trials testing combinations of ICI with other modalities are currently underway, including KEYNOTE-012 & 048 and CheckMate 141 & 358 (Ghosh et al., 2022; X. Zhou & Wang, 2022).

Cetuximab, known as a targeted and less toxic chemotherapeutic agent than cisplatin, has been widely tested for de-intensification chemotherapy strategies in the subset of HPV^+ HNSCC. It was approved for the treatment of HNSCC back in 2006, based on some promising results in combination with radiotherapy (Li et al., 2023). However, clinical studies such as RTOG 1016 (Gillison et al., 2019), De-ESCALaTE (Mehanna et al., 2019), and TROG12.01 (Deschuymer, Mehanna, & Nuyts, 2018) have rendered the judgement that cetuximab is unable to improve HPV^+ HNSCC patients' prognosis (Gillison et al., 2019; Sun et al., 2021). Studies have shown that viral oncoprotein E5 interacts with EGFR, upregulating the EGFR pathway (Ilahi & Bhatti, 2020), making EGFR antagonists ineffective in treating HPV infected cells. Another study attributed the chemoresistance of cetuximab to the ErbB protein and ligand mutations and aberrations of other downstream signaling components. (Ghosh et al., 2022; Yamaoka, Ohba, & Ohmori, 2017). Nantajit *et al.* has researched the disparate results from cetuximab in human vs. mice, with results pointing to a MAPK-independent pathway, the microRNA-9-5p and BRD4 axis. These researchers found that EGFR overexpression downregulates BRD4 and subsequently the oncoprotein E6 and E7 in HPV HNSCC. They suggested that rodents have a microRNA-9-5p that regulates the expression of BRD4 in tumor cells, leading to a better response in EGFR targeted tumor cells (Nantajit, Presta, Sauter, & Tavassoli, 2022). Such effects may

explain our findings of high efficacy in mice despite disappointing results from human trials.

Future Directions

We plan to continue studies that will validate or invalidate the viability of GA-OH as a clinical prospect. Our lab has demonstrated that GA-OH partnered with radiotherapy is effective in HPV^+ HNSCC (Chitsike et al., 2023). Testing this combination modality in a xenograft model would further corroborate the *in vitro* findings and create more treatment options for GA-OH in HNSCC patients. We would also consider other chemotherapeutic agents for HNSCC as combination partners in the future, such as carboplatin, another platinum-based therapy, 5-fluorouracil, or paclitaxel, which are all commonly used and recommended by the National Comprehensive Cancer Network (NCCN).

We also plan to extend our findings using patient derived samples to test the efficacy of GA-OH. Patient-derived primary samples would better represent the clinical situation than do cells from longer-term cultures, such as UM-SCC47. Furthermore, we would be able to own the intellectual and property rights for such a cell line if we proceeded to the clinical development of GA-OH. So far, our lab has successfully established an HPV-negative patient-derived cancer sample, and we are working to acquire and culture more HPV^+ cancer cells in the near future. Moreover, we have also established a cervical cancer xenograft model that can test the efficacy of GA-OH in an HPV-associated cancer other than HNSCC. If GA-OH continues to show efficacy in the other disease models, we would consider addressing HPV malignancies that are on the

rise, such as anal and vulvar (Cavallo, 2021; Liao et al., 2022). Efficacy across all types of cancers would broaden our spectrum and create an all-encompassing antiviral therapy for many HPV-associated tumors.

In terms of the mechanism of action of GA-OH, we are planning to examine changes in gene expression when HPV^+ and HPV^- cell lines are treated with GA-OH. If we continue to focus on pre-clinical development, one other question we want to answer includes an assessment of the activity of GA-OH against other subtypes of HPV. UM-SCC47 contains HPV type 16, which accounts for 80% of HNSCC (Ursu et al., 2022), and all experiments so far were tested on HPV-16 positive cell lines. Comparing effectiveness between HPV-18, which is the second most common type of HPV in HNSCC, and HPV-16 would elucidate the selectivity of GA-OH in these subtypes. Additionally, with the help of a medicinal chemist, we will consider optimizing GA-OH's structure for higher efficacy in the clinic. Developing antiviral small molecular therapy for HPV patients would ameliorate the many challenges that this subset of patients experience, as well as improve their recovery, quality of life, and therapeutical outcomes.

REFERENCES

Adams, A. K., Wise-Draper, T. M., & Wells, S. I. (2014). Human papillomavirus induced transformation in cervical and head and neck cancers. *Cancers (Basel), 6*(3), 1793-1820. doi:10.3390/cancers6031793

Aderhold, C., Faber, A., Grobschmidt, G. M., Chakraborty, A., Bockmayer, A., Umbreit, C., . . . Schultz, J. D. (2013). Small molecule-based chemotherapeutic approach in p16-positive and -negative HNSCC in vitro. *Anticancer Res, 33*(12), 5385-5393.

Aderhold, C., Faber, A., Umbreit, C., Chakraborty, A., Bockmayer, A., Birk, R., . . . Schultz, J. D. (2015). Small Molecules Alter VEGFR and PTEN Expression in HPV-positive and -negative SCC: New Hope for Targeted-therapy. *Anticancer Res, 35*(3), 1389-1399.

Ahn, S. M., Chan, J. Y., Zhang, Z., Wang, H., Khan, Z., Bishop, J. A., . . . Califano, J. A. (2014). Saliva and plasma quantitative polymerase chain reaction-based detection and surveillance of human papillomavirus-related head and neck cancer. *JAMA Otolaryngol Head Neck Surg, 140*(9), 846-854. doi:10.1001/jamaoto.2014.1338

Albers, A., Abe, K., Hunt, J., Wang, J., Lopez-Albaitero, A., Schaefer, C., . . . Ferris, R. L. (2005). Antitumor activity of human papillomavirus type 16 E7-specific T cells against virally infected squamous cell carcinoma of the head and neck. *Cancer Res, 65*(23), 11146-11155. doi:10.1158/0008-5472.can-05-0772

Almajhdi, F. N., Senger, T., Amer, H. M., Gissmann, L., & Ohlschlager, P. (2014). Design of a highly effective therapeutic HPV16 E6/E7-specific DNA vaccine: optimization by different ways of sequence rearrangements (shuffling). *PLoS One, 9*(11), e113461. doi:10.1371/journal.pone.0113461

Alsbeih, G., Al-Harbi, N., Bin Judia, S., Al-Qahtani, W., Khoja, H., El-Sebaie, M., & Tulbah, A. (2019). Prevalence of Human Papillomavirus (HPV) Infection and the Association with Survival in Saudi Patients with Head and Neck Squamous Cell Carcinoma. *Cancers (Basel), 11*(6). doi:10.3390/cancers11060820

Amine, A., Rivera, S., Opolon, P., Dekkal, M., Biard, D. S., Bouamar, H., . . . Vozenin-Brotons, M. C. (2009). Novel anti-metastatic action of cidofovir mediated by inhibition of E6/E7, CXCR4 and Rho/ROCK signaling in HPV tumor cells. *PLoS One, 4*(3), e5018. doi:10.1371/journal.pone.0005018

Amjad, M. T., Chidharla, A., & Kasi, A. (2023). Cancer Chemotherapy. In *StatPearls*. Treasure Island (FL).

Ang, K. K., Harris, J., Wheeler, R., Weber, R., Rosenthal, D. I., Nguyen-Tan, P. F., . . . Gillison, M. L. (2010). Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med, 363*(1), 24-35. doi:10.1056/NEJMoa0912217

Antonsson, A., Neale, R. E., Boros, S., Lampe, G., Coman, W. B., Pryor, D. I., . . . Whiteman, D. C. (2015). Human papillomavirus status and p16 expression in patients with mucosal squamous cell carcinoma of the head and neck in Queensland, Australia. *Cancer Epidemiol, 39*(2), 174-181. doi:10.1016/j.canep.2015.01.010

Arbyn, M., de Sanjose, S., Saraiya, M., Sideri, M., Palefsky, J., Lacey, C., . . . Monsonego, J. (2012). EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. *Int J Cancer, 131*(9), 1969-1982. doi:10.1002/ijc.27650

Argiris, A., Kotsakis, A. P., Hoang, T., Worden, F. P., Savvides, P., Gibson, M. K., . . . Kim, S. (2013). Cetuximab and bevacizumab: preclinical data and phase II trial in recurrent or metastatic squamous cell carcinoma of the head and neck. *Ann Oncol, 24*(1), 220-225. doi:10.1093/annonc/mds245

Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., . . . Schwall, R. H. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest, 104*(2), 155-162. doi:10.1172/jci6926

Ayuso, J. M., Vitek, R., Swick, A. D., Skala, M. C., Wisinski, K. B., Kimple, R. J., . . . Beebe, D. J. (2019). Effects of culture method on response to EGFR therapy in head and neck squamous cell carcinoma cells. *Sci Rep, 9*(1), 12480. doi:10.1038/s41598-019-48764-3

Baleja, J. D., Cherry, J. J., Liu, Z., Gao, H., Nicklaus, M. C., Voigt, J. H., . . . Androphy, E. J. (2006). Identification of inhibitors to papillomavirus type 16 E6 protein based on three-dimensional structures of interacting proteins. *Antiviral Res, 72*(1), 49-59. doi:10.1016/j.antiviral.2006.03.014

Band, V., De Caprio, J. A., Delmolino, L., Kulesa, V., & Sager, R. (1991). Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J Virol, 65*(12), 6671-6676.

Banik, K., Harsha, C., Bordoloi, D., Lalduhsaki Sailo, B., Sethi, G., Leong, H. C., . . . Kunnumakkara, A. B. (2018). Therapeutic potential of gambogic acid, a caged xanthone, to target cancer. *Cancer Lett, 416*, 75-86. doi:10.1016/j.canlet.2017.12.014

Beerheide, W., Bernard, H. U., Tan, Y. J., Ganesan, A., Rice, W. G., & Ting, A. E. (1999). Potential drugs against cervical cancer: zinc-ejecting inhibitors of the human papillomavirus type 16 E6 oncoprotein. *J Natl Cancer Inst, 91*(14), 1211-1220.

Beerheide, W., Sim, M. M., Tan, Y. J., Bernard, H. U., & Ting, A. E. (2000). Inactivation of the human papillomavirus-16 E6 oncoprotein by organic disulfides. *Bioorg Med Chem, 8*(11), 2549-2560.

Bellail, A. C., Qi, L., Mulligan, P., Chhabra, V., & Hao, C. (2009). TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges. *Rev Recent Clin Trials, 4*(1), 34-41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19149761

Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefebvre, J. L., Greiner, R. H., . . . 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. doi:10.1056/NEJMoa032641

Best, S. R., Niparko, K. J., & Pai, S. I. (2012). Biology of human papillomavirus infection and immune therapy for HPV-related head and neck cancers. *Otolaryngol Clin North Am, 45*(4), 807-822. doi:10.1016/j.otc.2012.04.005

Bishop, J. A., Lewis, J. S., Jr., Rocco, J. W., & Faquin, W. C. (2015). HPV-related squamous cell carcinoma of the head and neck: An update on testing in routine pathology practice. *Semin Diagn Pathol*. doi:10.1053/j.semdp.2015.02.013

Bishop, J. A., Ma, X. J., Wang, H., Luo, Y., Illei, P. B., Begum, S., . . . Westra, W. H. (2012). Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. *Am J Surg Pathol, 36*(12), 1874-1882. doi:10.1097/PAS.0b013e318265fb2b

Blioumi, E., Chatzidimitriou, D., Pazartzi, C., Katopodi, T., Tzimagiorgis, G., Emmanouil-Nikoloussi, E. N., . . . Antoniades, D. (2014). Detection and typing of human papillomaviruses (HPV) in malignant, dysplastic, nondysplastic and normal oral epithelium by nested polymerase chain reaction, immunohistochemistry and transitional electron microscopy in patients of northern Greece. *Oral Oncol, 50*(9), 840-847. doi:10.1016/j.oraloncology.2014.06.011

Blitzer, G. C., Smith, M. A., Harris, S. L., & Kimple, R. J. (2014). Review of the clinical and biologic aspects of human papillomavirus-positive squamous cell carcinomas of the head and neck. *Int J Radiat Oncol Biol Phys, 88*(4), 761-770. doi:10.1016/j.ijrobp.2013.08.029

Bol, V., & Gregoire, V. (2014). Biological basis for increased sensitivity to radiation therapy in HPV-positive head and neck cancers. *Biomed Res Int, 2014*, 696028. doi:10.1155/2014/696028

Bonilla-Velez, J., Mroz, E. A., Hammon, R. J., & Rocco, J. W. (2013). Impact of human papillomavirus on oropharyngeal cancer biology and response to therapy: implications for treatment. *Otolaryngol Clin North Am, 46*(4), 521-543. doi:10.1016/j.otc.2013.04.009

Bonner, J. A., Harari, P. M., Giralt, J., Azarnia, N., Shin, D. M., Cohen, R. B., . . . Ang, K. K. (2006). Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med, 354*(6), 567-578. doi:10.1056/NEJMoa053422

Bosch, F. X., Broker, T. R., Forman, D., Moscicki, A. B., Gillison, M. L., Doorbar, J., . . . de Sanjose, S. (2013). Comprehensive control of human papillomavirus infections and related diseases. *Vaccine, 31 Suppl 8*, I1-31. doi:10.1016/j.vaccine.2013.07.026

Boscolo-Rizzo, P., Del Mistro, A., Bussu, F., Lupato, V., Baboci, L., Almadori, G., . . . Paludetti, G. (2013). New insights into human papillomavirus-associated head and neck squamous cell carcinoma. *Acta Otorhinolaryngol Ital, 33*(2), 77-87.

Boyer, S. N., Wazer, D. E., & Band, V. (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res, 56*(20), 4620-4624.

Broglie, M. A., Soltermann, A., Rohrbach, D., Haile, S. R., Pawlita, M., Studer, G., . . . Stoeckli, S. J. (2013). Impact of p16, p53, smoking, and alcohol on survival in patients with oropharyngeal squamous cell carcinoma treated with primary intensity-modulated chemoradiation. *Head Neck, 35*(12), 1698-1706. doi:10.1002/hed.23231

Brown, C. E., Starr, R., Martinez, C., Aguilar, B., D'Apuzzo, M., Todorov, I., . . . Jensen, M. C. (2009). Recognition and killing of brain tumor stem-like initiating cells by CD8+ cytolytic T cells. *Cancer Res, 69*(23), 8886-8893. doi:10.1158/0008-5472.CAN-09-2687

Buchsbaum, D. J., Zhou, T., Grizzle, W. E., Oliver, P. G., Hammond, C. J., Zhang, S., . . . LoBuglio, A. F. (2003). Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res, 9*(10 Pt 1), 3731-3741. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14506165

Burtness, B., Bourhis, J. P., Vermorken, J. B., Harrington, K. J., & Cohen, E. E. (2014). Afatinib versus placebo as adjuvant therapy after chemoradiation in a double-blind, phase III study (LUX-Head & Neck 2) in patients with primary unresected, clinically intermediate-to-high-risk head and neck cancer: study protocol for a randomized controlled trial. *Trials, 15*, 469. doi:10.1186/1745-6215-15-469

Caicedo-Granados, E., Lin, R., Fujisawa, C., Yueh, B., Sangwan, V., & Saluja, A. (2014). Wild-type p53 reactivation by small-molecule Minnelide in human papillomavirus (HPV)-positive head and neck squamous cell carcinoma. *Oral Oncol, 50*(12), 1149-1156. doi:10.1016/j.oraloncology.2014.09.013

Cavallo, J. (2021). With the Exception of Cervical Cancer, HPV-Related Cancers Are on the Rise, Especially in Older Adults. Retrieved from https://ascopost.com/issues/june-25-2021/with-the-exception-of-cervical-cancer-hpv-related-cancers-are-on-the-rise/

Chai, R. C., Lambie, D., Verma, M., & Punyadeera, C. (2015). Current trends in the etiology and diagnosis of HPV-related head and neck cancers. *Cancer Med*. doi:10.1002/cam4.424

Chaturvedi, A. K., Anderson, W. F., Lortet-Tieulent, J., Curado, M. P., Ferlay, J., Franceschi, S., . . . Gillison, M. L. (2013). Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *J Clin Oncol, 31*(36), 4550-4559. doi:10.1200/jco.2013.50.3870

Chaturvedi, A. K., Engels, E. A., Pfeiffer, R. M., Hernandez, B. Y., Xiao, W., Kim, E., . . . Gillison, M. L. (2011). Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol, 29*(32), 4294-4301. doi:10.1200/jco.2011.36.4596

Cherry, J. J., Rietz, A., Malinkevich, A., Liu, Y., Xie, M., Bartolowits, M., . . . Androphy, E. J. (2013). Structure based identification and characterization of flavonoids that disrupt human papillomavirus-16 E6 function. *PLoS One, 8*(12), e84506. doi:10.1371/journal.pone.0084506

Chitsike, L., Bertucci, A., Vazquez, M., Lee, S., Unternaehrer, J. J., & Duerksen-Hughes, P. J. (2023). GA-OH enhances the cytotoxicity of photon and proton radiation in HPV(+) HNSCC cells. *Front Oncol, 13*, 1070485. doi:10.3389/fonc.2023.1070485

Chitsike, L., Yuan, C. H., Roy, A., Boyle, K., & Duerksen-Hughes, P. J. (2021). A high-content AlphaScreen identifies E6-specific small molecule inhibitors as potential therapeutics for HPV(+) head and neck squamous cell carcinomas. *Oncotarget, 12*(6), 549-561. doi:10.18632/oncotarget.27908

Cho, Y., Cho, C., Joung, O., Lee, K., Park, S., & Yoon, D. (2000). Development of screening systems for drugs against human papillomavirus-associated cervical cancer: based on E6-E6AP binding. *Antiviral Res, 47*(3), 199-206.

Choulier, L., Nomine, Y., Zeder-Lutz, G., Charbonnier, S., Didier, B., Jung, M. L., & Altschuh, D. (2013). Chemical library screening using a SPR-based inhibition in solution assay: simulations and experimental validation. *Anal Chem, 85*(18), 8787-8795. doi:10.1021/ac4019445

Chuntharapai, A., Dodge, K., Grimmer, K., Schroeder, K., Marsters, S. A., Koeppen, H., . . . Kim, K. J. (2001). Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J Immunol, 166*(8), 4891-4898. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11290766

Conte, E., Bresciani, E., Rizzi, L., Cappellari, O., De Luca, A., Torsello, A., & Liantonio, A. (2020). Cisplatin-Induced Skeletal Muscle Dysfunction: Mechanisms and Counteracting Therapeutic Strategies. *Int J Mol Sci, 21*(4). doi:10.3390/ijms21041242

Cooper, J. S., Pajak, T. F., Forastiere, A. A., Jacobs, J., Campbell, B. H., Saxman, S. B., . . . 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. doi:10.1056/NEJMoa032646

Cooper, T., Biron, V. L., Fast, D., Tam, R., Carey, T., Shmulevitz, M., & Seikaly, H. (2015). Oncolytic activity of reovirus in HPV positive and negative head and neck squamous cell carcinoma. *J Otolaryngol Head Neck Surg, 44*(1), 8. doi:10.1186/s40463-015-0062-x

Coppock, J. D., Wieking, B. G., Molinolo, A. A., Gutkind, J. S., Miskimins, W. K., & Lee, J. H. (2013). Improved clearance during treatment of HPV-positive head and neck cancer through mTOR inhibition. *Neoplasia, 15*(6), 620-630.

Cubie, H. A., & Cuschieri, K. (2013). Understanding HPV tests and their appropriate applications. *Cytopathology, 24*(5), 289-308. doi:10.1111/cyt.12083

D'Abramo, C. M., & Archambault, J. (2011). Small molecule inhibitors of human papillomavirus protein - protein interactions. *Open Virol J, 5*, 80-95. doi:10.2174/1874357901105010080

D'Souza, G., Agrawal, Y., Halpern, J., Bodison, S., & Gillison, M. L. (2009). Oral sexual behaviors associated with prevalent oral human papillomavirus infection. *J Infect Dis, 199*(9), 1263-1269. doi:10.1086/597755

D'Souza, G., & Dempsey, A. (2011). The role of HPV in head and neck cancer and review of the HPV vaccine. *Prev Med, 53 Suppl 1*, S5-s11. doi:10.1016/j.ypmed.2011.08.001

D'Souza, G., Kreimer, A. R., Viscidi, R., Pawlita, M., Fakhry, C., Koch, W. M., . . . Gillison, M. L. (2007). Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med, 356*(19), 1944-1956. doi:10.1056/NEJMoa065497

Dalianis, T. (2014a). Human papillomavirus (HPV) and oropharyngeal squamous cell carcinoma. *Presse Med, 43*(12p2), e429-e434. doi:10.1016/j.lpm.2014.08.010

Dalianis, T. (2014b). Human papillomavirus and oropharyngeal cancer, the epidemics, and significance of additional clinical biomarkers for prediction of response to therapy (Review). *Int J Oncol, 44*(6), 1799-1805. doi:10.3892/ijo.2014.2355

Damasio, M. P. S., Nascimento, C. S., Andrade, L. M., de Oliveira, V. L., & Calzavara-Silva, C. E. (2022). The role of T-cells in head and neck squamous cell carcinoma: From immunity to immunotherapy. *Front Oncol, 12*, 1021609. doi:10.3389/fonc.2022.1021609

De Miguel, D., Gallego-Lleyda, A., Ayuso, J. M., Erviti-Ardanaz, S., Pazo-Cid, R., del Agua, C., . . . Martinez-Lostao, L. (2016). TRAIL-coated lipid-nanoparticles overcome resistance to soluble recombinant TRAIL in non-small cell lung cancer cells. *Nanotechnology, 27*(18), 185101. doi:10.1088/0957-4484/27/18/185101

De Miguel, D., Gallego-Lleyda, A., Ayuso, J. M., Pawlak, A., Conde, B., Ochoa, I., . . . Martinez-Lostao, L. (2016). Improved Anti-Tumor Activity of Novel Highly Bioactive Liposome-Bound TRAIL in Breast Cancer Cells. *Recent Pat Anticancer Drug Discov, 11*(2), 197-214. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26924736

de Miguel, D., Lemke, J., Anel, A., Walczak, H., & Martinez-Lostao, L. (2016). Onto better TRAILs for cancer treatment. *Cell Death Differ, 23*(5), 733-747. doi:10.1038/cdd.2015.174

Deschuymer, S., Mehanna, H., & Nuyts, S. (2018). Toxicity Reduction in the Treatment of HPV Positive Oropharyngeal Cancer: Emerging Combined Modality Approaches. *Front Oncol, 8*, 439. doi:10.3389/fonc.2018.00439

Devaraj, K., Gillison, M. L., & Wu, T. C. (2003). Development of HPV vaccines for HPV-associated head and neck squamous cell carcinoma. *Crit Rev Oral Biol Med, 14*(5), 345-362.

Diana, G., & Corica, C. (2021). Human Papilloma Virus vaccine and prevention of head and neck cancer, what is the current evidence? *Oral Oncol, 115*, 105168. doi:10.1016/j.oraloncology.2020.105168

Dictor, M., & Warenholt, J. (2011). Single-tube multiplex PCR using type-specific E6/E7 primers and capillary electrophoresis genotypes 21 human papillomaviruses in neoplasia. *Infect Agent Cancer, 6*(1), 1. doi:10.1186/1750-9378-6-1

Dorsey, K., & Agulnik, M. (2013). Promising new molecular targeted therapies in head and neck cancer. *Drugs, 73*(4), 315-325. doi:10.1007/s40265-013-0025-3

Dreyer, J. H., Hauck, F., Oliveira-Silva, M., Barros, M. H., & Niedobitek, G. (2013). Detection of HPV infection in head and neck squamous cell carcinoma: a practical proposal. *Virchows Arch, 462*(4), 381-389. doi:10.1007/s00428-013-1393-5

Duek, I., Billan, S., Amit, M., & Gil, Z. (2014). Transoral Robotic Surgery in the HPV Era. *Rambam Maimonides Med J, 5*(2), e0010. doi:10.5041/rmmj.10144

Duenas-Gonzalez, A., Cetina, L., Coronel, J., & Cervantes-Madrid, D. (2012). Emerging drugs for cervical cancer. *Expert Opin Emerg Drugs, 17*(2), 203-218. doi:10.1517/14728214.2012.683409

Duiker, E. W., de Vries, E. G., Mahalingam, D., Meersma, G. J., Boersma-van Ek, W., Hollema, H., . . . de Jong, S. (2009). Enhanced antitumor efficacy of a DR5-specific TRAIL variant over recombinant human TRAIL in a bioluminescent ovarian cancer xenograft model. *Clin Cancer Res, 15*(6), 2048-2057. doi:10.1158/1078-0432.CCR-08-1535

Duncan, L. D., Winkler, M., Carlson, E. R., Heidel, R. E., Kang, E., & Webb, D. (2013). p16 immunohistochemistry can be used to detect human papillomavirus in oral cavity squamous cell carcinoma. *J Oral Maxillofac Surg, 71*(8), 1367-1375. doi:10.1016/j.joms.2013.02.019

Dyson, N., Howley, P. M., Munger, K., & Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science, 243*(4893), 934-937.

Elrefaey, S., Massaro, M. A., Chiocca, S., Chiesa, F., & Ansarin, M. (2014). HPV in oropharyngeal cancer: the basics to know in clinical practice. *Acta Otorhinolaryngol Ital, 34*(5), 299-309.

Fakhry, C., Westra, W. H., Li, S., Cmelak, A., Ridge, J. A., Pinto, H., . . . Gillison, M. L. (2008). Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst, 100*(4), 261-269. doi:10.1093/jnci/djn011

Fanger, N. A., Maliszewski, C. R., Schooley, K., & Griffith, T. S. (1999). Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med, 190*(8), 1155-1164.

Fera, D., Schultz, D. C., Hodawadekar, S., Reichman, M., Donover, P. S., Melvin, J., . . . Marmorstein, R. (2012). Identification and characterization of small molecule antagonists of pRb inactivation by viral oncoproteins. *Chem Biol, 19*(4), 518-528. doi:10.1016/j.chembiol.2012.03.007

Ferris, R. L., & Westra, W. (2023). Oropharyngeal Carcinoma with a Special Focus on HPV-Related Squamous Cell Carcinoma. *Annu Rev Pathol, 18*, 515-535. doi:10.1146/annurev-pathmechdis-031521-041424

Fertig, E. J., Markovic, A., Danilova, L. V., Gaykalova, D. A., Cope, L., Chung, C. H., . . . Califano, J. A. (2013). Preferential activation of the hedgehog pathway by epigenetic modulations in HPV negative HNSCC identified with meta-pathway analysis. *PLoS One, 8*(11), e78127. doi:10.1371/journal.pone.0078127

Filippova, M., Brown-Bryan, T. A., Casiano, C. A., & Duerksen-Hughes, P. J. (2005). The human papillomavirus 16 E6 protein can either protect or further sensitize cells to TNF: effect of dose. *Cell Death Differ, 12*(12), 1622-1635. doi:10.1038/sj.cdd.4401678

Filippova, M., Evans, W., Aragon, R., Filippov, V., Williams, V. M., Hong, L., . . . Duerksen-Hughes, P. (2014). The small splice variant of HPV16 E6, E6, reduces tumor formation in cervical carcinoma xenografts. *Virology, 450-451*, 153-164. doi:10.1016/j.virol.2013.12.011

Filippova, M., Filippov, V. A., Kagoda, M., Garnett, T., Fodor, N., & Duerksen-Hughes, P. J. (2009). Complexes of human papillomavirus type 16 E6 proteins form pseudo-death-inducing signaling complex structures during tumor necrosis factor-mediated apoptosis. *J Virol, 83*(1), 210-227. doi:10.1128/jvi.01365-08

Filippova, M., Johnson, M. M., Bautista, M., Filippov, V., Fodor, N., Tungteakkhun, S. S., . . . Duerksen-Hughes, P. J. (2007). The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity. *J Virol, 81*(8), 4116-4129. doi:10.1128/jvi.01924-06

Filippova, M., Parkhurst, L., & Duerksen-Hughes, P. J. (2004). The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J Biol Chem, 279*(24), 25729-25744. doi:10.1074/jbc.M401172200

Filippova, M., Song, H., Connolly, J. L., Dermody, T. S., & Duerksen-Hughes, P. J. (2002). The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J Biol Chem, 277*(24), 21730-21739. doi:10.1074/jbc.M200113200

Fonmarty, D., Cherriere, S., Fleury, H., Eimer, S., Majoufre-Lefebvre, C., Castetbon, V., & de Mones, E. (2015). Study of the concordance between p16 immunohistochemistry and HPV-PCR genotyping for the viral diagnosis of oropharyngeal squamous cell carcinoma. *Eur Ann Otorhinolaryngol Head Neck Dis*. doi:10.1016/j.anorl.2015.01.003

Forastiere, A. A., Goepfert, H., Maor, M., Pajak, T. F., Weber, R., Morrison, W., . . . Cooper, J. (2003). Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. *N Engl J Med, 349*(22), 2091-2098. doi:10.1056/NEJMoa031317

Ford, S. E., Brandwein-Gensler, M., Carroll, W. R., Rosenthal, E. L., & Magnuson, J. S. (2014). Transoral robotic versus open surgical approaches to oropharyngeal squamous cell carcinoma by human papillomavirus status. *Otolaryngol Head Neck Surg, 151*(4), 606-611. doi:10.1177/0194599814542939

Friedman, J. M., Stavas, M. J., & Cmelak, A. J. (2014). Clinical and scientific impact of human papillomavirus on head and neck cancer. *World J Clin Oncol, 5*(4), 781-791. doi:10.5306/wjco.v5.i4.781

Fumagalli, I., Dugue, D., Bibault, J. E., Clemenson, C., Vozenin, M. C., Mondini, M., & Deutsch, E. (2015). Cytotoxic effect of lapatinib is restricted to human papillomavirus-positive head and neck squamous cell carcinoma cell lines. *Onco Targets Ther, 8*, 335-345. doi:10.2147/ott.s68235

Galli, C. L., Allevi, P., Colombo, D., Corsini, E., Marinelli, P., Orlando, L., & Restani, P. (1989). Evaluation of the Oral Toxicity of Spinacine Hydrochloride in a 13-Week Study in Rats. *Food and Chemical Toxicology, 27*(10), 651-656. doi:Doi 10.1016/0278-6915(89)90119-1

Gao, G., Johnson, S. H., Kasperbauer, J. L., Eckloff, B. W., Tombers, N. M., Vasmatzis, G., & Smith, D. I. (2014). Mate pair sequencing of oropharyngeal squamous cell carcinomas reveals that HPV integration occurs much less frequently than in cervical cancer. *J Clin Virol, 59*(3), 195-200. doi:10.1016/j.jcv.2013.12.006

Garbuglia, A. R. (2014). Human papillomavirus in head and neck cancer. *Cancers (Basel), 6*(3), 1705-1726. doi:10.3390/cancers6031705

Garnett, T. O., Filippova, M., & Duerksen-Hughes, P. J. (2006). Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis. *Cell Death Differ, 13*(11), 1915-1926. doi:10.1038/sj.cdd.4401886

Garnett, T. O., Filippova, M., & Duerksen-Hughes, P. J. (2007). Bid is cleaved upstream of caspase-8 activation during TRAIL-mediated apoptosis in human osteosarcoma cells. *Apoptosis, 12*(7), 1299-1315. doi:10.1007/s10495-007-0058-8

Genden, E. M. (2012). The role for surgical management of HPV-related oropharyngeal carcinoma. *Head Neck Pathol, 6 Suppl 1*, S98-103. doi:10.1007/s12105-012-0362-7

George, M. (2014). Should patients with HPV-positive or negative tumors be treated differently? *Curr Oncol Rep, 16*(5), 384. doi:10.1007/s11912-014-0384-2

Ghosh, S., Shah, P. A., & Johnson, F. M. (2022). Novel Systemic Treatment Modalities Including Immunotherapy and Molecular Targeted Therapy for Recurrent and Metastatic Head and Neck Squamous Cell Carcinoma. *Int J Mol Sci, 23*(14). doi:10.3390/ijms23147889

Gillison, M. L., Broutian, T., Pickard, R. K., Tong, Z. Y., Xiao, W., Kahle, L., . . . Chaturvedi, A. K. (2012). Prevalence of oral HPV infection in the United States, 2009-2010. *Jama, 307*(7), 693-703. doi:10.1001/jama.2012.101

Gillison, M. L., Castellsague, X., Chaturvedi, A., Goodman, M. T., Snijders, P., Tommasino, M., . . . Franceschi, S. (2014). Eurogin Roadmap: comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. *Int J Cancer, 134*(3), 497-507. doi:10.1002/ijc.28201

Gillison, M. L., Koch, W. M., Capone, R. B., Spafford, M., Westra, W. H., Wu, L., . . . Sidransky, D. (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst, 92*(9), 709-720.

Gillison, M. L., Trotti, A. M., Harris, J., Eisbruch, A., Harari, P. M., Adelstein, D. 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. doi:10.1016/S0140-6736(18)32779-X

Goldstein, I., Rivlin, N., Shoshana, O. Y., Ezra, O., Madar, S., Goldfinger, N., & Rotter, V. (2013). Chemotherapeutic agents induce the expression and activity of their clearing enzyme CYP3A4 by activating p53. *Carcinogenesis, 34*(1), 190-198. doi:10.1093/carcin/bgs318

Grasso, F., Negri, D. R., Mochi, S., Rossi, A., Cesolini, A., Giovannelli, A., . . . Cara, A. (2013). Successful therapeutic vaccination with integrase defective lentiviral vector expressing nononcogenic human papillomavirus E7 protein. *Int J Cancer, 132*(2), 335-344. doi:10.1002/ijc.27676

Griffin, H., Elston, R., Jackson, D., Ansell, K., Coleman, M., Winter, G., & Doorbar, J. (2006). Inhibition of papillomavirus protein function in cervical cancer cells by intrabody targeting. *J Mol Biol, 355*(3), 360-378. doi:10.1016/j.jmb.2005.10.077

Griffith, T. S., Wiley, S. R., Kubin, M. Z., Sedger, L. M., Maliszewski, C. R., & Fanger, N. A. (1999). Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med, 189*(8), 1343-1354.

Guo, T., Qualliotine, J. R., Ha, P. K., Califano, J. A., Kim, Y., Saunders, J. R., . . . Fakhry, C. (2015). Surgical salvage improves overall survival for patients with HPV-positive and HPV-negative recurrent locoregional and distant metastatic oropharyngeal cancer. *Cancer*. doi:10.1002/cncr.29323

Haedicke, J., & Iftner, T. (2013). Human papillomaviruses and cancer. *Radiother Oncol, 108*(3), 397-402. doi:10.1016/j.radonc.2013.06.004

Hamoir, M., Ferlito, A., Schmitz, S., Hanin, F. X., Thariat, J., Weynand, B., . . . Takes, R. P. (2012). The role of neck dissection in the setting of chemoradiation therapy for head and neck squamous cell carcinoma with advanced neck disease. *Oral Oncol, 48*(3), 203-210. doi:10.1016/j.oraloncology.2011.10.015

Hatami, E., Jaggi, M., Chauhan, S. C., & Yallapu, M. M. (2020). Gambogic acid: A shining natural compound to nanomedicine for cancer therapeutics. *Biochim Biophys Acta Rev Cancer, 1874*(1), 188381. doi:10.1016/j.bbcan.2020.188381

Hemminki, K., Dong, C., & Frisch, M. (2000). Tonsillar and other upper aerodigestive tract cancers among cervical cancer patients and their husbands. *Eur J Cancer Prev, 9*(6), 433-437.

Hietanen, S., Lain, S., Krausz, E., Blattner, C., & Lane, D. P. (2000). Activation of p53 in cervical carcinoma cells by small molecules. *Proc Natl Acad Sci U S A, 97*(15), 8501-8506.

Hinni, M. L., Nagel, T., & Howard, B. (2015). Oropharyngeal cancer treatment: the role of transoral surgery. *Curr Opin Otolaryngol Head Neck Surg, 23*(2), 132-138. doi:10.1097/moo.0000000000000143

Hsu, H. W., Wall, N. R., Hsueh, C. T., Kim, S., Ferris, R. L., Chen, C. S., & Mirshahidi, S. (2014). Combination antiangiogenic therapy and radiation in head and neck cancers. *Oral Oncol, 50*(1), 19-26. doi:10.1016/j.oraloncology.2013.10.003

Huibregtse, J. M., Scheffner, M., & Howley, P. M. (1991). A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J, 10*(13), 4129-4135. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1661671

Husain, H., Psyrri, A., Markovic, A., Rampias, T., Pectasides, E., Wang, H., . . . Chung, C. H. (2012). Nuclear epidermal growth factor receptor and p16 expression in head and neck squamous cell carcinoma. *Laryngoscope, 122*(12), 2762-2768. doi:10.1002/lary.23647

Ichikawa, K., Liu, W., Zhao, L., Wang, Z., Liu, D., Ohtsuka, T., . . . Zhou, T. (2001). Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med, 7*(8), 954-960. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11479629

Ilahi, N. E., & Bhatti, A. (2020). Impact of HPV E5 on viral life cycle via EGFR signaling. *Microb Pathog, 139*, 103923. doi:10.1016/j.micpath.2019.103923

Inhestern, J., Oertel, K., Stemmann, V., Schmalenberg, H., Dietz, A., Rotter, N., . . . Guntinas-Lichius, O. (2015). Prognostic Role of Circulating Tumor Cells during Induction Chemotherapy Followed by Curative Surgery Combined with Postoperative Radiotherapy in Patients with Locally Advanced Oral and Oropharyngeal Squamous Cell Cancer. *PLoS One, 10*(7), e0132901. doi:10.1371/journal.pone.0132901

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L. G., Masucci, M., . . . Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med, 10*(12), 1321-1328. doi:10.1038/nm1146

Jarboe, E. A., Hunt, J. P., & Layfield, L. J. (2012). Cytomorphologic diagnosis and HPV testing of metastatic and primary oropharyngeal squamous cell carcinomas: a review and summary of the literature. *Diagn Cytopathol, 40*(6), 491-497. doi:10.1002/dc.22837

Jeon, S., Allen-Hoffmann, B. L., & Lambert, P. F. (1995). Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J Virol, 69*(5), 2989-2997.

Jeremias, I., Herr, I., Boehler, T., & Debatin, K. M. (1998). TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *Eur J Immunol, 28*(1), 143-152. doi:10.1002/(sici)1521-4141(199801)28:01<143::aid-immu143>3.0.co;2-3

Jia, B., Li, S., Hu, X., Zhu, G., & Chen, W. (2015). Recent research on bioactive xanthones from natural medicine: Garcinia hanburyi. *AAPS PharmSciTech, 16*(4), 742-758. doi:10.1208/s12249-015-0339-4

John, K., Wu, J., Lee, B. W., & Farah, C. S. (2013). MicroRNAs in Head and Neck Cancer. *Int J Dent, 2013*, 650218. doi:10.1155/2013/650218

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. *Nat Rev Dis Primers, 6*(1), 92. doi:10.1038/s41572-020-00224-3

Johnsson, A., & Wennerberg, J. (1999). Amifostine as a protector against cisplatin-induced toxicity in nude mice. *Acta Oncol, 38*(2), 247-253. doi:10.1080/028418699431681

Kaczmar, J. M., Tan, K. S., Heitjan, D. F., Lin, A., Ahn, P. H., Newman, J. G., . . . Weinstein, G. S. (2014). HPV-related oropharyngeal cancer: Risk factors for treatment failure in patients managed with primary transoral robotic surgery. *Head Neck*. doi:10.1002/hed.23850

Kayagaki, N., Yamaguchi, N., Nakayama, M., Kawasaki, A., Akiba, H., Okumura, K., & Yagita, H. (1999). Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. *J Immunol, 162*(5), 2639-2647.

Kelesidis, T., Aish, L., Steller, M. A., Aish, I. S., Shen, J., Foukas, P., . . . Tsiodras, S. (2011). Human papillomavirus (HPV) detection using in situ hybridization in histologic samples: correlations with cytologic changes and polymerase chain reaction HPV detection. *Am J Clin Pathol, 136*(1), 119-127. doi:10.1309/ajcp03huqyzmwatp

Kennedy, E. M., Kornepati, A. V., Goldstein, M., Bogerd, H. P., Poling, B. C., Whisnant, A. W., . . . Cullen, B. R. (2014). Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *J Virol, 88*(20), 11965-11972. doi:10.1128/jvi.01879-14

Khanal, S., Shumway, B. S., Zahin, M., Redman, R. A., Strickley, J. D., Trainor, P. J., . . . Joh, J. (2018). Viral DNA integration and methylation of human papillomavirus type 16 in high-grade oral epithelial dysplasia and head and neck squamous cell carcinoma. *Oncotarget, 9*(54), 30419-30433. doi:10.18632/oncotarget.25754

Kim, E. J., & Wierzbicki, A. S. (2021). Investigating raised creatine kinase. *BMJ, 373*, n1486. doi:10.1136/bmj.n1486

Kimple, A. J., Torres, A. D., Yang, R. Z., & Kimple, R. J. (2012). HPV-associated head and neck cancer: molecular and nano-scale markers for prognosis and therapeutic stratification. *Sensors (Basel), 12*(4), 5159-5169. doi:10.3390/s120405159

Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., & Ashkenazi, A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity, 12*(6), 611-620.

Koskinen, W. J., Chen, R. W., Leivo, I., Makitie, A., Back, L., Kontio, R., . . . Aaltonen, L. M. (2003). Prevalence and physical status of human papillomavirus in squamous cell carcinomas of the head and neck. *Int J Cancer, 107*(3), 401-406. doi:10.1002/ijc.11381

Kreimer, A. R. (2014). Prospects for prevention of HPV-driven oropharynx cancer. *Oral Oncol, 50*(6), 555-559. doi:10.1016/j.oraloncology.2013.06.007

Krishnamurthy, S., Ahmed, I., Bhise, R., Mohanti, B. K., Sharma, A., Rieckmann, T., . . . Bonomo, P. (2022). The dogma of Cetuximab and Radiotherapy in head and neck cancer - A dawn to dusk journey. *Clin Transl Radiat Oncol, 34*, 75-81. doi:10.1016/j.ctro.2022.03.009

Kumar, B., Cordell, K. G., Lee, J. S., Prince, M. E., Tran, H. H., Wolf, G. T., . . . Carey, T. E. (2007). Response to therapy and outcomes in oropharyngeal cancer are associated with biomarkers including human papillomavirus, epidermal growth factor receptor, gender, and smoking. *Int J Radiat Oncol Biol Phys, 69*(2 Suppl), S109-111. doi:10.1016/j.ijrobp.2007.05.072

Kumar, B., Cordell, K. G., Lee, J. S., Worden, F. P., Prince, M. E., Tran, H. H., . . . Carey, T. E. (2008). EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. *J Clin Oncol, 26*(19), 3128-3137. doi:10.1200/jco.2007.12.7662

Kumar, S., Biswas, M., & Jose, T. (2015). HPV vaccine: Current status and future directions. *Med J Armed Forces India, 71*(2), 171-177. doi:10.1016/j.mjafi.2015.02.006

Kwon, J., Yoon, H. J., Kim, J. H., Lee, T. S., Song, I. H., Lee, H. W., . . . Park, J. H. (2014). Cetuximab inhibits cisplatin-induced activation of EGFR signaling in esophageal squamous cell carcinoma. *Oncol Rep, 32*(3), 1188-1192. doi:10.3892/or.2014.3302

Langer, C. J. (2012). Exploring biomarkers in head and neck cancer. *Cancer, 118*(16), 3882-3892. doi:10.1002/cncr.26718

Langevin, S. M., Grandis, J. R., & Taioli, E. (2011). Female hormonal and reproductive factors and head and neck squamous cell carcinoma risk. *Cancer Lett, 310*(2), 216-221. doi:10.1016/j.canlet.2011.07.007

Laskar, S. G., & Swain, M. (2015). HPV positive oropharyngeal cancer and treatment deintensification: How pertinent is it? *J Cancer Res Ther, 11*(1), 6-9. doi:10.4103/0973-1482.151445

Lechner, M., Frampton, G. M., Fenton, T., Feber, A., Palmer, G., Jay, A., . . . Boshoff, C. (2013). Targeted next-generation sequencing of head and neck squamous cell

carcinoma identifies novel genetic alterations in HPV+ and HPV- tumors. *Genome Med, 5*(5), 49. doi:10.1186/gm453

Lechner, M. S., & Laimins, L. A. (1994). Inhibition of p53 DNA binding by human papillomavirus E6 proteins. *J Virol, 68*(7), 4262-4273.

Li, Q., Tie, Y., Alu, A., Ma, X., & Shi, H. (2023). Targeted therapy for head and neck cancer: signaling pathways and clinical studies. *Signal Transduct Target Ther, 8*(1), 31. doi:10.1038/s41392-022-01297-0

Liao, C. I., Francoeur, A. A., Kapp, D. S., Caesar, M. A. P., Huh, W. K., & Chan, J. K. (2022). Trends in Human Papillomavirus-Associated Cancers, Demographic Characteristics, and Vaccinations in the US, 2001-2017. *JAMA Netw Open, 5*(3), e222530. doi:10.1001/jamanetworkopen.2022.2530

Lin, K., Doolan, K., Hung, C. F., & Wu, T. C. (2010). Perspectives for preventive and therapeutic HPV vaccines. *J Formos Med Assoc, 109*(1), 4-24.

Linxweiler, M., Bochen, F., Wemmert, S., Lerner, C., Hasenfus, A., Bohle, R. M., . . . Schick, B. (2015). Combination of p16(INK4a) /Ki67 immunocytology and hpv polymerase chain reaction for the noninvasive analysis of HPV involvement in head and neck cancer. *Cancer Cytopathol, 123*(4), 219-229. doi:10.1002/cncy.21512

Liu, W., Ning, R., Chen, R. N., Hu, J. H., Gui, H. Y., Wang, Y. W., . . . Guo, Q. L. (2015). Gambogic acid suppresses cytochrome P450 3A4 by downregulating pregnane X receptor and up-regulating DEC1 in human hepatoma HepG2 cells. *Toxicology Research, 4*(4), 1059-1071. doi:10.1039/c4tx00239c

Liu, X., Clements, A., Zhao, K., & Marmorstein, R. (2006). Structure of the human Papillomavirus E7 oncoprotein and its mechanism for inactivation of the retinoblastoma tumor suppressor. *J Biol Chem, 281*(1), 578-586. doi:10.1074/jbc.M508455200

Lohaus, F., Linge, A., Tinhofer, I., Budach, V., Gkika, E., Stuschke, M., . . . Baumann, M. (2014). HPV16 DNA status is a strong prognosticator of loco-regional control after postoperative radiochemotherapy of locally advanced oropharyngeal carcinoma: results from a multicentre explorative study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG). *Radiother Oncol, 113*(3), 317-323. doi:10.1016/j.radonc.2014.11.011

Lorincz, B. B., Jowett, N., & Knecht, R. (2015). Decision management in transoral robotic surgery (tors): Indications, individual patient selection, and role in the multidisciplinary treatment of head and neck cancer from a european perspective. *Head Neck*. doi:10.1002/hed.24059

Luedke, E., Jaime-Ramirez, A. C., Bhave, N., Roda, J., Choudhary, M. M., Kumar, B., . . . Carson, W. E., 3rd. (2012). Cetuximab therapy in head and neck cancer: immune modulation with interleukin-12 and other natural killer cell-activating cytokines. *Surgery, 152*(3), 431-440. doi:10.1016/j.surg.2012.05.035

Lui, V. W., & Grandis, J. R. (2012). Primary chemotherapy and radiation as a treatment strategy for HPV-positive oropharyngeal cancer. *Head Neck Pathol, 6 Suppl 1*, S91-97. doi:10.1007/s12105-012-0364-5

Ma, B., Maraj, B., Tran, N. P., Knoff, J., Chen, A., Alvarez, R. D., . . . Wu, T. C. (2012). Emerging human papillomavirus vaccines. *Expert Opin Emerg Drugs, 17*(4), 469-492. doi:10.1517/14728214.2012.744393

Machiels, J. P., Lambrecht, M., Hanin, F. X., Duprez, T., Gregoire, V., Schmitz, S., & Hamoir, M. (2014). Advances in the management of squamous cell carcinoma of the head and neck. *F1000Prime Rep, 6*, 44. doi:10.12703/p6-44

Mahal, B. A., Catalano, P. J., Haddad, R. I., Hanna, G. J., Kass, J. I., Schoenfeld, J. D., . . . Margalit, D. N. (2019). Incidence and Demographic Burden of HPV-Associated Oropharyngeal Head and Neck Cancers in the United States. *Cancer Epidemiol Biomarkers Prev, 28*(10), 1660-1667. doi:10.1158/1055-9965.EPI-19-0038

Malecka, K. A., Fera, D., Schultz, D. C., Hodawadekar, S., Reichman, M., Donover, P. S., . . . Marmorstein, R. (2014). Identification and characterization of small molecule human papillomavirus E6 inhibitors. *ACS Chem Biol, 9*(7), 1603-1612. doi:10.1021/cb500229d

Malhotra, B., Bellile, E. L., Nguyen, N. P., Fung, V. K., Slack, M., Bilich, R., . . . Worden, F. (2014). Carboplatin-pemetrexed in treatment of patients with recurrent/metastatic cancers of the head and neck; superior outcomes in oropharyngeal primaries. *Front Oncol, 4*, 362. doi:10.3389/fonc.2014.00362

Mannarini, L., Kratochvil, V., Calabrese, L., Gomes Silva, L., Morbini, P., Betka, J., & Benazzo, M. (2009). Human Papilloma Virus (HPV) in head and neck region: review of literature. *Acta Otorhinolaryngol Ital, 29*(3), 119-126.

Martinez-Useros, J., & Garcia-Foncillas, J. (2015). The challenge of blocking a wider family members of EGFR against head and neck squamous cell carcinomas. *Oral Oncol*. doi:10.1016/j.oraloncology.2015.02.092

Masterson, L., Moualed, D., Liu, Z. W., Howard, J. E., Dwivedi, R. C., Tysome, J. R., . . . Goon, P. K. (2014). De-escalation treatment protocols for human papillomavirus-associated oropharyngeal squamous cell carcinoma: a systematic review and meta-analysis of current clinical trials. *Eur J Cancer, 50*(15), 2636-2648. doi:10.1016/j.ejca.2014.07.001

Maxwell, J. H., Mehta, V., Wang, H., Cunningham, D., Duvvuri, U., Kim, S., . . . Ferris, R. L. (2014). Quality of life in head and neck cancer patients: impact of HPV and primary treatment modality. *Laryngoscope, 124*(7), 1592-1597. doi:10.1002/lary.24508

McBride, S. M., Parambi, R. J., Jang, J. W., Goldsmith, T., Busse, P. M., & Chan, A. W. (2014). Intensity-modulated versus conventional radiation therapy for oropharyngeal carcinoma: long-term dysphagia and tumor control outcomes. *Head Neck, 36*(4), 492-498. doi:10.1002/hed.23319

McDonald, S. A., Qendri, V., Berkhof, J., de Melker, H. E., & Bogaards, J. A. (2017). Disease burden of human papillomavirus infection in the Netherlands, 1989-2014: the gap between females and males is diminishing. *Cancer Causes Control*. doi:10.1007/s10552-017-0870-6

Mehanna, H., Robinson, M., Hartley, A., Kong, A., Foran, B., Fulton-Lieuw, T., . . . De, E. H. P. V. T. G. (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. doi:10.1016/S0140-6736(18)32752-1

Meistrich, M. L. (2013). Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertil Steril, 100*(5), 1180-1186. doi:10.1016/j.fertnstert.2013.08.010

Melkane, A. E., Mirghani, H., Auperin, A., Saulnier, P., Lacroix, L., Vielh, P., . . . Temam, S. (2014). HPV-related oropharyngeal squamous cell carcinomas: a comparison between three diagnostic approaches. *Am J Otolaryngol, 35*(1), 25-32. doi:10.1016/j.amjoto.2013.08.007

Mirghani, H., Amen, F., Blanchard, P., Moreau, F., Guigay, J., Hartl, D. M., & Lacau St Guily, J. (2015). Treatment de-escalation in HPV-positive oropharyngeal carcinoma: ongoing trials, critical issues and perspectives. *Int J Cancer, 136*(7), 1494-1503. doi:10.1002/ijc.28847

Mirghani, H., & Blanchard, P. (2018). Treatment de-escalation for HPV-driven oropharyngeal cancer: Where do we stand? *Clin Transl Radiat Oncol, 8*, 4-11. doi:10.1016/j.ctro.2017.10.005

Modur, V., Thomas-Robbins, K., & Rao, K. (2015). HPV and CSC in HNSCC cisplatin resistance. *Front Biosci (Elite Ed), 7*, 58-66.

Mongkolsapaya, J., Grimes, J. M., Chen, N., Xu, X. N., Stuart, D. I., Jones, E. Y., & Screaton, G. R. (1999). Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. *Nat Struct Biol, 6*(11), 1048-1053. doi:10.1038/14935

Monie, A., Tsen, S. W., Hung, C. F., & Wu, T. C. (2009). Therapeutic HPV DNA vaccines. *Expert Rev Vaccines, 8*(9), 1221-1235. doi:10.1586/erv.09.76

Monnier, Y., & Simon, C. (2015). Surgery Versus Radiotherapy for Early Oropharyngeal Tumors: a Never-Ending Debate. *Curr Treat Options Oncol, 16*(9), 362. doi:10.1007/s11864-015-0362-4

Moore, K. A., 2nd, & Mehta, V. (2015). The Growing Epidemic of HPV-Positive Oropharyngeal Carcinoma: A Clinical Review for Primary Care Providers. *J Am Board Fam Med, 28*(4), 498-503. doi:10.3122/jabfm.2015.04.140301

Moscicki, A. B. (2005). Impact of HPV infection in adolescent populations. *J Adolesc Health, 37*(6 Suppl), S3-9. doi:10.1016/j.jadohealth.2005.09.011

Mott, F. E., Sacks, R., Johnson, F., Hutcheson, K. A., Gallagher, N., Varghese, S., & Zaveri, J. (2020). Subjective functional outcomes in oropharyngeal cancer treated with induction chemotherapy using the MD Anderson Symptom Inventory (MDASI). *Laryngoscope Investig Otolaryngol, 5*(6), 1104-1109. doi:10.1002/lio2.487

Mydlarz, W. K., Chan, J. Y., & Richmon, J. D. (2015). The role of surgery for HPV-associated head and neck cancer. *Oral Oncol, 51*(4), 305-313. doi:10.1016/j.oraloncology.2014.10.005

Nantajit, D., Presta, L., Sauter, T., & Tavassoli, M. (2022). EGFR-induced suppression of HPV E6/E7 is mediated by microRNA-9-5p silencing of BRD4 protein in HPV-positive head and neck squamous cell carcinoma. *Cell Death Dis, 13*(11), 921. doi:10.1038/s41419-022-05269-8

Ndiaye, C., Mena, M., Alemany, L., Arbyn, M., Castellsague, X., Laporte, L., . . . Trottier, H. (2014). HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. *Lancet Oncol, 15*(12), 1319-1331. doi:10.1016/S1470-2045(14)70471-1

Nelke, K. H., Lysenko, L., Leszczyszyn, J., & Gerber, H. (2013). Human papillomavirus and its influence on head and neck cancer predisposition. *Postepy Hig Med Dosw (Online), 67*, 610-616.

Nichols, A. C., Yoo, J., Hammond, J. A., Fung, K., Winquist, E., Read, N., . . . Palma, D. A. (2013). Early-stage squamous cell carcinoma of the oropharynx: radiotherapy vs. trans-oral robotic surgery (ORATOR)--study protocol for a randomized phase II trial. *BMC Cancer, 13*, 133. doi:10.1186/1471-2407-13-133

Nicol, C., Cesur, O., Forrest, S., Belyaeva, T. A., Bunka, D. H., Blair, G. E., & Stonehouse, N. J. (2013). An RNA aptamer provides a novel approach for the induction of apoptosis by targeting the HPV16 E7 oncoprotein. *PLoS One, 8*(5), e64781. doi:10.1371/journal.pone.0064781

O'Leary, P., & Kjaergaard, T. (2014). Transoral robotic surgery and oropharyngeal cancer: a literature review. *Ear Nose Throat J, 93*(8), E14-21.

Oikonomou, E., & Pintzas, A. (2013). The TRAIL of oncogenes to apoptosis. *Biofactors, 39*(4), 343-354. doi:10.1002/biof.1112

Olthof, N. C., Huebbers, C. U., Kolligs, J., Henfling, M., Ramaekers, F. C., Cornet, I., . . . Speel, E. J. (2015). Viral load, gene expression and mapping of viral integration sites in HPV16-associated HNSCC cell lines. *Int J Cancer, 136*(5), E207-218. doi:10.1002/ijc.29112

Osazuwa-Peters, N., Wang, D. D., Namin, A., Vivek, J., O'Neill, M., Patel, P. V., & Varvares, M. A. (2015). Sexual behavior, HPV knowledge, and association with head and neck cancer among a high-risk group. *Oral Oncol*. doi:10.1016/j.oraloncology.2015.01.011

Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., & Dixit, V. M. (1997). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science, 277*(5327), 815-818.

Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., & Dixit, V. M. (1997). The receptor for the cytotoxic ligand TRAIL. *Science, 276*(5309), 111-113.

Parkin, D. M. (2006). The global health burden of infection-associated cancers in the year 2002. *Int J Cancer, 118*(12), 3030-3044. doi:10.1002/ijc.21731

Patel, S. N., Cohen, M. A., Givi, B., Dixon, B. J., Gilbert, R. W., Gullane, P. J., . . . Goldstein, D. P. (2015). Salvage surgery of locally recurrent oropharyngeal cancer. *Head Neck*. doi:10.1002/hed.24065

Pellegrino, L., & Resmini, P. (1996). Evaluation of the stable reaction products of histidine with formaldehyde or with other carbonyl compounds in dairy products. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 202*(1), 66-71. doi:Doi 10.1007/Bf01229688

Pickard, R. K., Xiao, W., Broutian, T. R., He, X., & Gillison, M. L. (2012). The prevalence and incidence of oral human papillomavirus infection among young men and women, aged 18-30 years. *Sex Transm Dis, 39*(7), 559-566. doi:10.1097/OLQ.0b013e31824f1c65

Pinkiewicz, M., Dorobisz, K., & Zatonski, T. (2022). Human Papillomavirus-Associated Head and Neck Cancers. Where are We Now? A Systematic Review. *Cancer Manag Res, 14*, 3313-3324. doi:10.2147/CMAR.S379173

Psyrri, A., Sasaki, C., Vassilakopoulou, M., Dimitriadis, G., & Rampias, T. (2012). Future directions in research, treatment and prevention of HPV-related squamous cell

carcinoma of the head and neck. *Head Neck Pathol, 6 Suppl 1*, S121-128. doi:10.1007/s12105-012-0361-8

Psyrri, A., Seiwert, T. Y., & Jimeno, A. (2013). Molecular pathways in head and neck cancer: EGFR, PI3K, and more. *Am Soc Clin Oncol Educ Book*, 246-255. doi:10.1200/EdBook_AM.2013.33.246

Purohit, S., Bhise, R., Lokanatha, D., & Govindbabu, K. (2013). Systemic therapy in head and neck cancer: changing paradigm. *Indian J Surg Oncol, 4*(1), 19-26. doi:10.1007/s13193-012-0197-4

Qi, Q., You, Q., Gu, H., Zhao, L., Liu, W., Lu, N., & Guo, Q. (2008). Studies on the toxicity of gambogic acid in rats. *J Ethnopharmacol, 117*(3), 433-438. doi:10.1016/j.jep.2008.02.027

Ramshankar, V., & Krishnamurthy, A. (2013). Human papilloma virus in head and neck cancers-role and relevance in clinical management. *Indian J Surg Oncol, 4*(1), 59-66. doi:10.1007/s13193-012-0196-5

Rein, D. T., & Kurbacher, C. M. (2001). The role of chemotherapy in invasive cancer of the cervix uteri: current standards and future prospects. *Anticancer Drugs, 12*(10), 787-795.

Restani, P., Campagner, P., Fiecchi, A., Resmini, P., & Galli, C. L. (1988). Identification of Spinacine as the Principal Reaction-Product of Gamma-Casein with Formaldehyde in Cheese. *Food and Chemical Toxicology, 26*(5), 441-446. doi:Doi 10.1016/0278-6915(88)90055-5

Rettig, E., Kiess, A. P., & Fakhry, C. (2015). The role of sexual behavior in head and neck cancer: implications for prevention and therapy. *Expert Rev Anticancer Ther, 15*(1), 35-49. doi:10.1586/14737140.2015.957189

Reusser, N. M., Downing, C., Guidry, J., & Tyring, S. K. (2015). HPV Carcinomas in Immunocompromised Patients. *J Clin Med, 4*(2), 260-281. doi:10.3390/jcm4020260

Ridge, J. A. (2014). Surgery in the HPV era: the role of robotics and microsurgical techniques. *Am Soc Clin Oncol Educ Book*, 154-159. doi:10.14694/EdBook_AM.2014.34.154

Riechelmann, H., Dejaco, D., Steinbichler, T. B., Lettenbichler-Haug, A., Anegg, M., Ganswindt, U., . . . Riedl, D. (2022). Functional Outcomes in Head and Neck Cancer Patients. *Cancers (Basel), 14*(9). doi:10.3390/cancers14092135

Rischin, D., Young, R. J., Fisher, R., Fox, S. B., Le, Q. T., Peters, L. J., . . . McArthur, G. A. (2010). Prognostic significance of p16INK4A and human papillomavirus in

patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. *J Clin Oncol, 28*(27), 4142-4148. doi:10.1200/jco.2010.29.2904

Roman, B. B., Wieringa, B., & Koretsky, A. P. (1997). Functional equivalence of creatine kinase isoforms in mouse skeletal muscle. *J Biol Chem, 272*(28), 17790-17794. doi:10.1074/jbc.272.28.17790

Sabatini, M. E., & Chiocca, S. (2020). Human papillomavirus as a driver of head and neck cancers. *Br J Cancer, 122*(3), 306-314. doi:10.1038/s41416-019-0602-7

Sanders, A. E., Slade, G. D., & Patton, L. L. (2012). National prevalence of oral HPV infection and related risk factors in the U.S. adult population. *Oral Dis, 18*(5), 430-441. doi:10.1111/j.1601-0825.2011.01892.x

Sato, K., Hida, S., Takayanagi, H., Yokochi, T., Kayagaki, N., Takeda, K., . . . Ogasawara, K. (2001). Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol, 31*(11), 3138-3146. doi:10.1002/1521-4141(200111)31:11<3138::aid-immu3138>3.0.co;2-b

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., & Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell, 75*(3), 495-505. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8221889

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., & Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell, 63*(6), 1129-1136.

Scott-Wittenborn, N., D'Souza, G., Tewari, S., Rooper, L., Troy, T., Drake, V., . . . Fakhry, C. (2022). Prevalence of human papillomavirus in head and neck cancers at tertiary care centers in the United States over time. *Cancer, 128*(9), 1767-1774. doi:10.1002/cncr.34124

Scripture, C. D., Sparreboom, A., & Figg, W. D. (2005). Modulation of cytochrome P450 activity: implications for cancer therapy. *Lancet Oncol, 6*(10), 780-789. doi:10.1016/S1470-2045(05)70388-0

Sepiashvili, L., Bruce, J. P., Huang, S. H., O'Sullivan, B., Liu, F. F., & Kislinger, T. (2015). Novel Insights into Head and Neck Cancer using Next-Generation "Omic" Technologies. *Cancer Res, 75*(3), 480-486. doi:10.1158/0008-5472.can-14-3124

Settle, K., Posner, M. R., Schumaker, L. M., Tan, M., Suntharalingam, M., Goloubeva, O., . . . Cullen, K. J. (2009). Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal

cancer patients. *Cancer Prev Res (Phila), 2*(9), 776-781. doi:10.1158/1940-6207.capr-09-0149

Shaikh, F., Sanehi, P., & Rawal, R. (2012). Molecular screening of compounds to the predicted Protein-Protein Interaction site of Rb1-E7 with p53- E6 in HPV. *Bioinformation, 8*(13), 607-612. doi:10.6026/97320630008607

Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., . . . Ashkenazi, A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science, 277*(5327), 818-821.

Shi, W., Kato, H., Perez-Ordonez, B., Pintilie, M., Huang, S., Hui, A., . . . Liu, F. F. (2009). Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *J Clin Oncol, 27*(36), 6213-6221. doi:10.1200/jco.2009.23.1670

Smeets, S. J., Hesselink, A. T., Speel, E. J., Haesevoets, A., Snijders, P. J., Pawlita, M., . . . Brakenhoff, R. H. (2007). A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer, 121*(11), 2465-2472. doi:10.1002/ijc.22980

Smith, E. M., Ritchie, J. M., Summersgill, K. F., Klussmann, J. P., Lee, J. H., Wang, D., . . . Turek, L. P. (2004). Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *Int J Cancer, 108*(5), 766-772. doi:10.1002/ijc.11633

Smukste, I., Bhalala, O., Persico, M., & Stockwell, B. R. (2006). Using small molecules to overcome drug resistance induced by a viral oncogene. *Cancer Cell, 9*(2), 133-146. doi:10.1016/j.ccr.2006.01.012

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. doi:10.1016/j.semcancer.2018.01.008

Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., . . . Walczak, H. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity, 12*(6), 599-609.

Stanley, M. A. (2012). Genital human papillomavirus infections: current and prospective therapies. *J Gen Virol, 93*(Pt 4), 681-691. doi:10.1099/vir.0.039677-0

Steinau, M., Hariri, S., Gillison, M. L., Broutian, T. R., Dunne, E. F., Tong, Z. Y., . . . Unger, E. R. (2014). Prevalence of cervical and oral human papillomavirus infections among US women. *J Infect Dis, 209*(11), 1739-1743. doi:10.1093/infdis/jit799

Steiner, P., Joynes, C., Bassi, R., Wang, S., Tonra, J. R., Hadari, Y. R., & Hicklin, D. J. (2007). Tumor growth inhibition with cetuximab and chemotherapy in non-small cell lung cancer xenografts expressing wild-type and mutated epidermal growth factor receptor. *Clin Cancer Res, 13*(5), 1540-1551. doi:10.1158/1078-0432.CCR-06-1887

Stern, P. L., van der Burg, S. H., Hampson, I. N., Broker, T. R., Fiander, A., Lacey, C. J., . . . Einstein, M. H. (2012). Therapy of human papillomavirus-related disease. *Vaccine, 30 Suppl 5*, F71-82. doi:10.1016/j.vaccine.2012.05.091

Stuckey, D. W., & Shah, K. (2013). TRAIL on trial: preclinical advances in cancer therapy. *Trends Mol Med, 19*(11), 685-694. doi:10.1016/j.molmed.2013.08.007

S1471-4914(13)00154-8 [pii]

Sun, Y., Wang, Z., Qiu, S., & Wang, R. (2021). Therapeutic strategies of different HPV status in Head and Neck Squamous Cell Carcinoma. *Int J Biol Sci, 17*(4), 1104-1118. doi:10.7150/ijbs.58077

Surucu, M., Shah, K. K., Mescioglu, I., Roeske, J. C., Small, W., Jr., Choi, M., & Emami, B. (2015). Decision Trees Predicting Tumor Shrinkage for Head and Neck Cancer: Implications for Adaptive Radiotherapy. *Technol Cancer Res Treat*. doi:10.1177/1533034615572638

Syrjanen, K. J., Pyrhonen, S., Syrjanen, S. M., & Lamberg, M. A. (1983). Immunohistochemical demonstration of human papilloma virus (HPV) antigens in oral squamous cell lesions. *Br J Oral Surg, 21*(2), 147-153.

Szymonowicz, K. A., & Chen, J. (2020). Biological and clinical aspects of HPV-related cancers. *Cancer Biol Med, 17*(4), 864-878. doi:10.20892/j.issn.2095-3941.2020.0370

Tanaka, S., Sugimachi, K., Shirabe, K., Shimada, M., & Wands, J. R. (2000). Expression and antitumor effects of TRAIL in human cholangiocarcinoma. *Hepatology, 32*(3), 523-527. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10960444

Tang, A. L., Hauff, S. J., Owen, J. H., Graham, M. P., Czerwinski, M. J., Park, J. J., . . . Prince, M. E. (2012). UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. *Head Neck, 34*(10), 1480-1491. doi:10.1002/hed.21962

Trivedi, R., & Mishra, D. P. (2015). Trailing TRAIL Resistance: Novel Targets for TRAIL Sensitization in Cancer Cells. *Front Oncol, 5*, 69. doi:10.3389/fonc.2015.00069

Trosman, S. J., Koyfman, S. A., Ward, M. C., Al-Khudari, S., Nwizu, T., Greskovich, J. F., . . . Burkey, B. B. (2015). Effect of Human Papillomavirus on Patterns of Distant Metastatic Failure in Oropharyngeal Squamous Cell Carcinoma Treated With Chemoradiotherapy. *JAMA Otolaryngol Head Neck Surg*. doi:10.1001/jamaoto.2015.136

Tungteakkhun, S. S., & Duerksen-Hughes, P. J. (2008). Cellular binding partners of the human papillomavirus E6 protein. *Arch Virol, 153*(3), 397-408. doi:10.1007/s00705-007-0022-5

Tungteakkhun, S. S., Filippova, M., Fodor, N., & Duerksen-Hughes, P. J. (2010). The full-length isoform of human papillomavirus 16 E6 and its splice variant E6* bind to different sites on the procaspase 8 death effector domain. *J Virol, 84*(3), 1453-1463. doi:10.1128/jvi.01331-09

Tungteakkhun, S. S., Filippova, M., Neidigh, J. W., Fodor, N., & Duerksen-Hughes, P. J. (2008). The interaction between human papillomavirus type 16 and FADD is mediated by a novel E6 binding domain. *J Virol, 82*(19), 9600-9614. doi:10.1128/jvi.00538-08

Turner, D. O., Williams-Cocks, S. J., Bullen, R., Catmull, J., Falk, J., Martin, D., . . . Kingsley, K. (2011). High-risk human papillomavirus (HPV) screening and detection in healthy patient saliva samples: a pilot study. *BMC Oral Health, 11*, 28. doi:10.1186/1472-6831-11-28

Urban, D., Corry, J., & Rischin, D. (2014). What is the best treatment for patients with human papillomavirus-positive and -negative oropharyngeal cancer? *Cancer, 120*(10), 1462-1470. doi:10.1002/cncr.28595

Ursu, R. G., Luchian, I., Damian, C., Porumb-Andrese, E., Ghetu, N., Cobzaru, R. G., . . . Iancu, L. S. (2022). Diagnostics of HNSCC Patients: An Analysis of Cell Lines and Patient-Derived Xenograft Models for Personalized Therapeutical Medicine. *Diagnostics (Basel), 12*(5). doi:10.3390/diagnostics12051071

van Kempen, P. M., Noorlag, R., Braunius, W. W., Stegeman, I., Willems, S. M., & Grolman, W. (2014). Differences in methylation profiles between HPV-positive and HPV-negative oropharynx squamous cell carcinoma: a systematic review. *Epigenetics, 9*(2), 194-203. doi:10.1007/s13193-012-0196-5

Vermeer, D. W., Spanos, W. C., Vermeer, P. D., Bruns, A. M., Lee, K. M., & Lee, J. H. (2013). Radiation-induced loss of cell surface CD47 enhances immune-mediated clearance of human papillomavirus-positive cancer. *Int J Cancer, 133*(1), 120-129. doi:10.1002/ijc.28015

Vogt, S. L., Gravitt, P. E., Martinson, N. A., Hoffmann, J., & D'Souza, G. (2013). Concordant Oral-Genital HPV Infection in South Africa Couples: Evidence for Transmission. *Front Oncol, 3*, 303. doi:10.3389/fonc.2013.00303

Wang, L. H., Li, Y., Yang, S. N., Wang, F. Y., Hou, Y., Cui, W., . . . Wu, C. F. (2014). Gambogic acid synergistically potentiates cisplatin-induced apoptosis in non-small-cell lung cancer through suppressing NF-kappaB and MAPK/HO-1 signalling. *Br J Cancer, 110*(2), 341-352. doi:10.1038/bjc.2013.752

Ward, G., Mehta, V., & Moore, M. (2016). Morbidity, mortality and cost from HPV-related oropharyngeal cancer: Impact of 2-, 4- and 9-valent vaccines. *Hum Vaccin Immunother, 12*(6), 1343-1347. doi:10.1080/21645515.2015.1095415

Wazer, D. E., Liu, X. L., Chu, Q., Gao, Q., & Band, V. (1995). Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. *Proc Natl Acad Sci U S A, 92*(9), 3687-3691.

Weinberger, P. M., Merkley, M. A., Khichi, S. S., Lee, J. R., Psyrri, A., Jackson, L. L., & Dynan, W. S. (2010). Human papillomavirus-active head and neck cancer and ethnic health disparities. *Laryngoscope, 120*(8), 1531-1537. doi:10.1002/lary.20984

Weiss, D., Heinkele, T., & Rudack, C. (2015). Reliable detection of Human papillomavirus in recurrent laryngeal papillomatosis and associated carcinoma of archival tissue. *J Med Virol, 87*(5), 860-870. doi:10.1002/jmv.24124

Widdice, L. E., Breland, D. J., Jonte, J., Farhat, S., Ma, Y., Leonard, A. C., & Moscicki, A. B. (2010). Human papillomavirus concordance in heterosexual couples. *J Adolesc Health, 47*(2), 151-159. doi:10.1016/j.jadohealth.2010.01.006

Wierzbicka, M., Jozefiak, A., Szydlowski, J., Marszalek, A., Stankiewicz, C., Hassman-Poznanska, E., . . . Szyfter, W. (2013). Recommendations for the diagnosis of human papilloma virus (HPV) high and low risk in the prevention and treatment of diseases of the oral cavity, pharynx and larynx. Guide of experts PTORL and KIDL. *Otolaryngol Pol, 67*(3), 113-134. doi:10.1016/j.otpol.2013.01.003

Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., . . . et al. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity, 3*(6), 673-682.

Wittekindt, C., Wagner, S., & Klussmann, J. P. (2011). [HPV-associated head and neck cancer. The basics of molecular and translational research]. *Hno, 59*(9), 885-892. doi:10.1007/s00106-011-2357-1

Xie, X., Piao, L., Bullock, B. N., Smith, A., Su, T., Zhang, M., . . . Pan, Q. (2014). Targeting HPV16 E6-p300 interaction reactivates p53 and inhibits the tumorigenicity of

HPV-positive head and neck squamous cell carcinoma. *Oncogene, 33*(8), 1037-1046. doi:10.1038/onc.2013.25

Yamaoka, T., Ohba, M., & Ohmori, T. (2017). Molecular-Targeted Therapies for Epidermal Growth Factor Receptor and Its Resistance Mechanisms. *Int J Mol Sci, 18*(11). doi:10.3390/ijms18112420

Yan, F., Cowell, L. G., Tomkies, A., & Day, A. T. (2023). Therapeutic Vaccination for HPV-Mediated Cancers. *Curr Otorhinolaryngol Rep, 11*(1), 44-61. doi:10.1007/s40136-023-00443-8

Yazbeck, V. Y., Li, C., Grandis, J. R., Zang, Y., & Johnson, D. E. (2014). Single-agent obatoclax (GX15-070) potently induces apoptosis and pro-survival autophagy in head and neck squamous cell carcinoma cells. *Oral Oncol, 50*(2), 120-127. doi:10.1016/j.oraloncology.2013.10.013

Yi, C., Maksimoska, J., Marmorstein, R., & Kissil, J. L. (2010). Development of small-molecule inhibitors of the group I p21-activated kinases, emerging therapeutic targets in cancer. *Biochem Pharmacol, 80*(5), 683-689. doi:10.1016/j.bcp.2010.03.012

Younes, A., & Kadin, M. E. (2003). Emerging applications of the tumor necrosis factor family of ligands and receptors in cancer therapy. *J Clin Oncol, 21*(18), 3526-3534. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12972530

Yu, T., Ferber, M. J., Cheung, T. H., Chung, T. K., Wong, Y. F., & Smith, D. I. (2005). The role of viral integration in the development of cervical cancer. *Cancer Genet Cytogenet, 158*(1), 27-34. doi:10.1016/j.cancergencyto.2004.08.021

Yuan, C. H., Filippova, M., & Duerksen-Hughes, P. (2012). Modulation of apoptotic pathways by human papillomaviruses (HPV): mechanisms and implications for therapy. *Viruses, 4*(12), 3831-3850. doi:10.3390/v4123831

Yuan, C. H., Filippova, M., Krstenansky, J. L., & Duerksen-Hughes, P. J. (2016). Flavonol and imidazole derivatives block HPV16 E6 activities and reactivate apoptotic pathways in HPV(+) cells. *Cell Death Dis, 7*, 2060. doi:10.1038/cddis.2015.391

Yuan, C. H., Filippova, M., Tungteakkhun, S. S., Duerksen-Hughes, P. J., & Krstenansky, J. L. (2012). Small molecule inhibitors of the HPV16-E6 interaction with caspase 8. *Bioorg Med Chem Lett, 22*(5), 2125-2129. doi:10.1016/j.bmcl.2011.12.145

Zamai, L., Ahmad, M., Bennett, I. M., Azzoni, L., Alnemri, E. S., & Perussia, B. (1998). Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas

ligand by immature and mature primary human NK cells. *J Exp Med, 188*(12), 2375-2380.

Zhang, P., Mirani, N., Baisre, A., & Fernandes, H. (2014). Molecular heterogeneity of head and neck squamous cell carcinoma defined by next-generation sequencing. *Am J Pathol, 184*(5), 1323-1330. doi:10.1016/j.ajpath.2014.01.028

Zhao, C. Y., Szekely, L., Bao, W., & Selivanova, G. (2010). Rescue of p53 function by small-molecule RITA in cervical carcinoma by blocking E6-mediated degradation. *Cancer Res, 70*(8), 3372-3381. doi:10.1158/0008-5472.can-09-2787

Zhou, J. Z., Jou, J., & Cohen, E. (2021). Vaccine Strategies for Human Papillomavirus-Associated Head and Neck Cancers. *Cancers (Basel), 14*(1). doi:10.3390/cancers14010033

Zhou, X., & Wang, X. (2022). Radioimmunotherapy in HPV-Associated Head and Neck Squamous Cell Carcinoma. *Biomedicines, 10*(8). doi:10.3390/biomedicines10081990

Zorzi, S. F., Agostini, G., Chu, F., Tagliabue, M., Pietrobon, G., Corrao, G., . . . Ansarin, M. (2022). Upfront transoral robotic surgery (TORS) versus intensity-modulated radiation therapy (IMRT) in HPV-positive oropharyngeal cancer: real-world data from a tertiary comprehensive cancer centre. *Acta Otorhinolaryngol Ital, 42*(4), 334-347. doi:10.14639/0392-100X-N2144