A Combination of Three Synthetic Compounds Display Potent Antitumor Effects

By

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A Combination of Three Synthetic Compounds Derived from Plants Display Potent Antitumor Effects

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Abstract

Patients suffering from head and neck squamous cell carcinoma (HNSCC) encounter low survival while managing current aggressive therapies that cause significant morbidity. Nutraceuticals are diet-derived compounds with few known side effects. However, limited antitumor efficacy has restricted their use for cancer therapy. In this work, we examined combined plant compounds, established a combination therapy that was more potent than its singular components, and delineated its mechanism of action. Curcumin is a yellow polyphenol that is derived from the rhizome of turmeric (Curcuma longa). It is known to influence multiple cellular processes. Likewise, harmine, a beta-carboline alkaloid found in Peganum harmala, and isovanillin, a phenolic aldehyde isomer of vanilla, have shown antitumor activity. We tested the antitumor efficacy of three formulation: GZ17-S (combined plant extracts from *Arum palaestinum,* R *Peganum harmala* and *Curcuma longa*); GZ17-05.00 (16 synthetic components of GZ17-S); and GZ17-06.02 (chemically synthesized curcumin, harmine and isovanillin). We tested the formulations on HNSCC progression. Specifically, we carried out *in vitro* studies on HNSCC proliferation, migration, invasion, angiogenesis, apoptosis, and macrophage viability and infiltration into the tumor. We demonstrated that GZ17-06.02 was most effective in attenuating *in vitro* parameters of HNSCC progression. Further, GZ17-06.02 potentiated the efficacy of cisplatin and radiation therapy. We demonstrate that GZ17-06.02 has multiple molecular targets including EGFR, ERK1/2, and AKT. We used molecular docking analyses to validate that GZ17-06.02 components bound at distinct binding sites on these targets. Finally, we demonstrated that GZ17-06.02 significantly inhibited the growth of established HNSCC cell lines, patient-derived xenografts, and murine syngeneic tumors ($P<0.001$). We demonstrated GZ17-06.02 as a highly effective plant extract combination for HNSCC. These studies provide rationale for clinical testing of GZ17-06.02 for HNSCC and other solid malignancies.

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Chapter I: Introduction

Head and neck squamous cell carcinoma (HNSCC) is an illness that is severely burdensome, causing great morbidity and mortality. Globally, 500,000 new individuals are diagnosed every year [1]. Worldwide and domestically the incidence of HNSCC has persistently remained unchanged over the past three decades. The gravity of this disease is exemplified by the five-year survival rate that remains below 50% [2]. The current treatment mainstays are surgery, chemotherapy, and radiotherapy. While these treatment strategies can be effective, they often leave the patient to bear significant morbidity. The heavy cost of treatment demands better, less enervating therapeutic options.

Histologically, head and neck cancers originate from the mucosal surfaces of the larynx, pharynx and oral cavity. Approximately, 90% occur from proliferative squamous cells (HNSCC). The other 10% of head and neck cancers originate from salivary glands, and an even smaller percentage consists of sarcomas [3], paragangliomas [4], and neuroblastomas [5]. Not only is HNSCC the most common head and neck cancer, it is also the most lethal.

The risk factors of HNSCC are tobacco and alcohol consumption, which have been commonly associated with the disease. Increasingly, human papilloma virus (HPV) associated HNSCC is on the rise. The risk of HNSCC is dramatically increased with greater than 30 g ethanol/day, which is comparable to three “standard” drinks [6]) (multivariable adjusted incidence rate ratio: 6.39) [7]. Tobacco use increases the risk of hypo/oropharyngeal caner and laryngeal cancer (multivariable adjusted incidence rate

ratio: 8.53 and 8.07, respectively)[7]. As the smoking rates are declining globally, there has been a substantial decrease of non-HPV oropharyngeal carcinoma by 50% since the late 80's [8].

However, there has been a two-fold increase in HPV positive oropharyngeal carcinoma cases in the same time period [8]. Associative HPV factors include higher number of lifetime sexual partners and marijuana use, but not alcohol or tobacco usage [9]. Squamous epithelium of the head and neck, anus, and cervix are especially susceptible to HPV.

The oncogenic types HPV-16, HPV-18, HPV-31, and HPV-33 are sexually transmitted viruses that can transform infected cells.

Type 16 HPV are responsible for the vast majority of HPV related HNSCC. In most individuals, the virus clears. However, in those patients that fail to mount an adequate immune response, HPV a non-enveloped, DNA virus incorporates into the host genome, transforming the host cell with the E6 and E7 proteins that inhibit tumor suppressor, p53 and retinoblastoma (Rb), respectively[10]. Additionally, p16 binds and inhibits cyclin dependent kinase 4 and 6, thereby blocking the catalytic activity of CDK-cyclin D1 that are required for Rb phosphorylation; this initiates cell cycle arrest [11]. These tumors present differently and have better clinical outcomes that do carcinogen-induced HNSCC. However, HPV vaccines are 90-100% effective in preventing HPV infections that lead to precancerous lesions [12]. Thus, global HPV related cancers are projected to be dramatically reduced by the year 2050 [13].

Globally, other factors that contribute to HNSCC development include naswar (a mix of tobacco, calcium hydroxide, ash of plants, spices and oil) sniffing/dipping, Betel

(Areca) nut chewing, Khat chewing, and Maté [14]. These factors are all dose dependent and have been shown to have mutagenic properties. Finally, the Epstein Barr virus can induce cellular proliferation of the tonsil, oropharynx, and salivary gland. The nasopharynx is the commonly affected [15]. Nevertheless, HPV, tobacco, and alcohol comprise the vast majority inciting factors.

Figure 1-1: HPV Pathway of Cellular Transformation in HNSCC

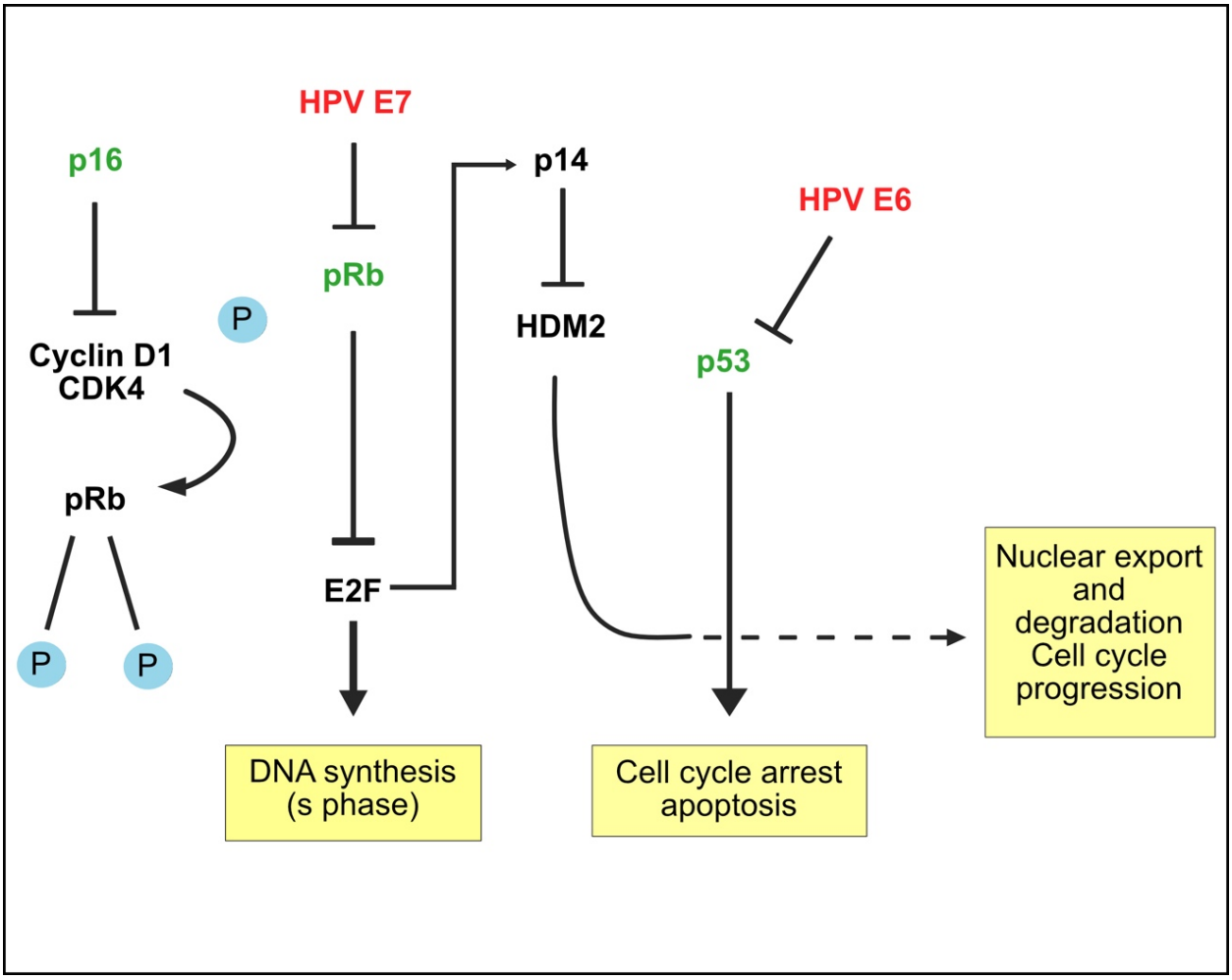

Figure 1-1: HPV cellularly transforms head and neck cancer cells. Following HPV infection, the viral oncoproteins E7 and E6 alter pRb and p53 respectively. Alteration of pRb inhibits E2F, a tumor suppressing transcription factor, leading to DNA synthesis. E2F alteration leads to increased p14, which then alters the production of HDM2, a ubiquitin ligase that suppress the p53. This in turn yield nuclear export and degradation of tumor suppressor and cell cycle progression. Inhibition of p53 directly by the E6 oncoprotein causes cell cycle arrest and apoptosis.

HNSCC patients are usually above the age of 40, from unspecified socioeconomic backgrounds, and have a higher male to female ratio of 3:1 (HPV negative); 8:1 (HPV positive) [16]. In North America 56% of HNSCC cases are HPV positive [17]. HPV-positive HNSCC patients are characteristically middle aged, non-smoking white men from higher socioeconomic status and have a history of multiple sexual partners [18].

Patients often present with lesions in the oral cavity that are self-discovered or clinically uncovered. Most of these lesions are easily noticed during examination. Thickening of the mucosa, a painful or painless mass, or ulcer accompanied by odynophagia and dysphagia are common clinical manifestations. However, lesions of the larynx are much more difficult to discover, in part because of their hard-to-self-visualize location in the body. In the larynx, patients often present with shortness of breath, hoarseness, stridor, and dysphagia [19].

Tumor staging, tobacco use, and race are all considered when prognosis is determined at initial diagnosis [20]. The American Joint Committee on Cancer has laid forth the HNSCC tumor staging guidelines. Prognosis is broken into categories ranging from best prognosis, 0, and worst, IV, depending on tumor (T) characteristics, nodal (N) characteristics, and distant metastasis (M). HPV positive HNSCC is specially considered using p16 immunohistochemistry (IHC) [21].

Other factors that influence prognosis are race and environment. Black non-Hispanics and present tobacco users have much worse outcomes [20]. Nonetheless, early detection provides better outcomes to patients. However, at initial diagnosis, over 40% of patients have advanced disease with regional nodal involvement [16].

HNSCC initiates with an intraepithelial proliferation of squamous cells. This cellular proliferation may become evident as a white patch or plaque (leukoplakia) that cannot be easily removed by rubbing [22].

HNSCC is a highly recurrent disease. Up to 30% of patients develop distant metastases, while the majority of patients develop some form of recurrent disease [23]. Field cancerization in HNSCC is caused by carcinogenic risk factors. This field cancerization causes the development of multiple primary tumors in the same anatomic region [24]. In concurrence with this phenomenon, epithelia surrounding the carcinoma undergoes genetic alterations [25]. Notwithstanding, targeted radiotherapy and sufficiently resected tumors do not account for the dysplastic field of carcinogen exposure, which facilitates a high rate of recurrence.

The five-year survival rate of HNSCC patients upon initial diagnosis is less than 50%. Even such poor outcomes demand a multidisciplinary approach including head and neck surgeons, medical oncologists, radiation oncologists, with support from speech therapists, social workers, psychologists, and dieticians.

Current Therapy

Patient characteristics, site, and stage are determinant factors that influence the use of the three treatment modalities of chemotherapy, radiation, and surgery. In early disease stage, surgery and radiation are the primary treatments. Chemotherapy is used in combination with other modalities across multiple disease stages. Surgery has had the best treatment outcome for many years which lead to the development of the surgeon’s stature as leader of the care team. However, microscopic disease that is not easily assessed or treated with surgery are treated with chemotherapy and radiation.

Radiotherapy is the primary treatment for HPV+ tumors. Each treatment option offers distinct advantages alongside distinct morbidities.

Surgery

For nearly all head and neck tumors, surgery provides the best control of the disease. Surgical technique and approach varies depending on tumor location and local aggressiveness [26]. Retaining function and cosmetic outcome are surgical considerations. Reconstructive surgery has advanced in the last 25 years, offering surgeons more latitude in establishing free margins [23]. Patients are more likely to experience minimal functional impacts with negative margins and proper reconstruction.

However, as the extent of the surgical procedure increases, as does the morbidity of the patient. Immediate risks following surgery are hemorrhage, aspiration pneumonia, and pulmonary embolism [23]. Following surgery, a pain management is implemented. Advanced disease usually requires large surgical margins, which can lead to disfiguration. Secondary reconstructive surgery may follow initial tumor resection. For early stage patients, the single modality of surgery can provide a cure rate of up to 90% [23].

Radiotherapy

Ionizing radiation damages cells directly through ionizing of target molecules and indirectly by generating reactive oxygen species from water [27]. In early stage HNSCC, single modality radiotherapy is often effective and can yield cure rates between 70-90% [28]. Nevertheless, late stage disease does not have such cure rates. Advanced disease can expect only 50% tumor control with radiotherapy alone, leaving the five-year survival rate at just 30% [28]. Side effects of radiation therapy are xerostomia, radiation-induced fibrosis, trismus, and osteoradionecrosis [29].

Chemotherapy

The last four decades have seen a substantial development of chemotherapy treatment for head and neck patients. Cisplatin was approved in 1978, which gave physicians options beyond the once standard palliative treatment with bleomycin and methotrexate [30, 31]. While there was a tumoral response from bleomycin and methotrexate, these drugs offered no improvement in overall survival. Alternatively, while cisplatin alone did not improve overall survival or response rate [32], it did confer the benefit of in combination treatments [33]. Cisplatin improves median survival by 1-2 months compared to supportive care alone [34]. Cisplatin in combination with taxanes or fluorouracil improve response rate of non-targeted chemotherapies [35]. Three decades after cisplatin was introduced, targeted EGFR therapy cetuximab, a monoclonal antibody, added another 2 months to the overall survival of cytotoxic therapy [36].

Immunotherapy has been the only area of improved overall survival in head and neck cancer patients since the approval of cetuximab over 10 years ago. The T-cell suppressive receptor, programmed death-1 (PD-1), is activated by programmed death ligands (PD-L1 and PD-L2) of the head and neck tumor cells, thereby evading the immune system [37]. While numerous clinical predictive diagnostics are currently under investigation, PD-L1 positivity testing by immunohistochemistry is widely accepted in head and neck cancer. This testing can be onerous as only membranous tumor tissues are considered significant, which may not be readily available following surgery[38]. For certain patients, nivolumab and pembrolizumab, both monoclonal antibodies targeting PD-1, improved overall survival [39, 40]. These targeted therapies might indicate an

avenue of further scientific exploration that will improve the lives of head and neck cancer patients.

Early stage disease is easily treated with surgery and radiation with good outcomes. However, advanced stage tumors generally require surgery, radiation, and chemotherapy. If the disease progresses, PD-1 targeted immunotherapies can be a second-line treatment. While these therapies are borne of more than five decades of scientific work, the best care will only improve overall survival by months, not years. Currently successful treatment, especially in later stages, requires a high morbidity cost. Likewise, chemotherapeutic agents currently available to treat head and neck cancer are highly toxic, destroy off-target cells, and cause the patient further illness. There is a current need for agents that are efficacious, cause few side effects, and are generally well tolerated by patients.

Chapter II: Plant Compounds as Head and Neck Treatment

A brief history of food as medicine

Nearly 5000 years ago the oldest written evidence of plants being used as medicine was recorded on a Sumerian clay slab from Nagpur. The slab described a 12 medicinal recipes containing over 250 plants, some of them were alkaloid like poppy, mandrake, and henbane [41]. Empedocles of Agrigetum (490-430 B.C.E.) was the first to introduce the idea that balanced food consumption could treat disease, leading to humoral theory [41]. Hippocrates of Kos, the "Father of Medicine," while being the first major proponent of medicine as an independent profession, implementing the study of clinical medicine, and summarizing medical knowledge of previous schools is also credited with the phrase "Let food be thy medicine, and medicine be thy food"[42]. Indeed, early medical practitioners in the same vein were known to adjust diet based on the illness presented. Although an aphorism, the successful treatment of many patients in ancient medicine was likely due to meeting dietary inadequacies, not a direct interaction between food and disease [43]. The first century Greek surgeon Galen interpreted the work of Hippocrates in his practice and training. He implemented Hippocrates' humoral theory (the belief that the body contained "humors", and that if not in balance would produce disease and poor temperament [44]), prescribing food depending on the humor responsible for the affliction [45]. Galen's practice and teaching influenced medicine for the next 1,300 years [46]. Humoral theory was not seriously challenged until the 16th century by physicians. In 1628 William Harvey published *De Motu Cordus* (Latin for "An Anatomical Exercise on the Motion of the Heard and Blood in Living Beings"), which came in direct conflict with humoral theory and centuries of Galenic medicine [47]. In 1858, Rudolf Virchow published *Cellular Pathology* [48],

known as the first systematic pathology text, that contributed to cell theory and ended humoral theory entirely, and brought the decline of food as medicine.

Following the rise of the industrial revolution in the West, food as medicine slipped out of medicine and into the fringes of alternative medicine, and lacked general acceptance in the medical profession [49]. However, following the discovery and classification of vitamins, food and naturally derived compounds were beginning to gain attention once again in the science community [50]. In the field of cancer, the discovery of paclitaxel in 1962 moved forward treatment options for many types of cancer. Paclitaxel, a microtubule binding agent, was initially harvested from the bark of yew trees [51]. Vincristine, another tubule targeting drug, was first isolated in 1961 from flowering *Vinca* plants. Again this added to the regime of drugs available to treat Hodgkin's lymphoma and non-Hodgkin's lymphoma [52]. As production of chemotherapies slowed during the latter half of the 20th century, an interest in anecdotal evidence in non-Western treatments of cancer rose [53]. Eastern medicine, middle east folk medicine, and Ayurvedic medicine commonly prescribe many roots, seeds, and barks based treatments [53].

Plants Compounds and Head and Neck Cancer

In 1989 Stephen D. Felice coined the term nutraceutical (a portmanteau of nutrition and pharmaceutical) in an effort to more systematically study and classify plant and food based medicine [54]. Nutraceutical is broadly defined as any substance that can be considered a food or part of a food and provides medical or health benefits including the treatment and prevention of disease [53]. Nutraceuticals have long been considered safe and generally acceptable. Some of the first nutraceuticals to be tested in the treatment of HNSCC with antitumor efficacy include genistein [55], resveratrol [56],

luteolin [57], and isothiocyanate [58]. Indeed, lycopene [59], vitamin A derivatives [31], green tea extract [60], curcumin [61], and soybean extract have all undergone HNSCC clinical trials [62]. These studies are limited in that they are preventive studies, not treatment of the disease directly.

There are no previous studies investigating a combination of natural compounds in the treatment of HNSCC. Combining cancer treatment agents is proven to reduce side effects of single agents and have additive anticancer effects. This spurred interest in investigating combinations of nutraceuticals in the treatment of HNSCC, which may allow for improved efficacy and lower drug concentrations.

Curcumin

Curcuma longa is a heavily studied plant[63]. Curcumin, the active ingredient, inhibits nuclear factor-κB (NF-κB), epidermal growth factor receptor (EGFR), mitogen activated protein kinase (MAPK), and vascular endothelial growth factor (VEGF) (Fig 2-1) [64-66]. However, curcumin also has poor bioavailability [67]. This has developed interest in curcumin analogs and nanoparticle encapsulation to increase bioavailability [68]. Additionally, combining curcumin with different nutraceuticals potentiates efficacy [69], and the combination of curcumin provides additive benefit to chemotherapeutics [70]. Crooker et al reviewed natural compounds in the treatment of HNSCC with particular focus on curcumin. HNSCC is shown to have multiple targets affected by various nutraceuticals (Fig 2-2) [66].

Figure 2-1: Targets of Curcumin in HNSCC

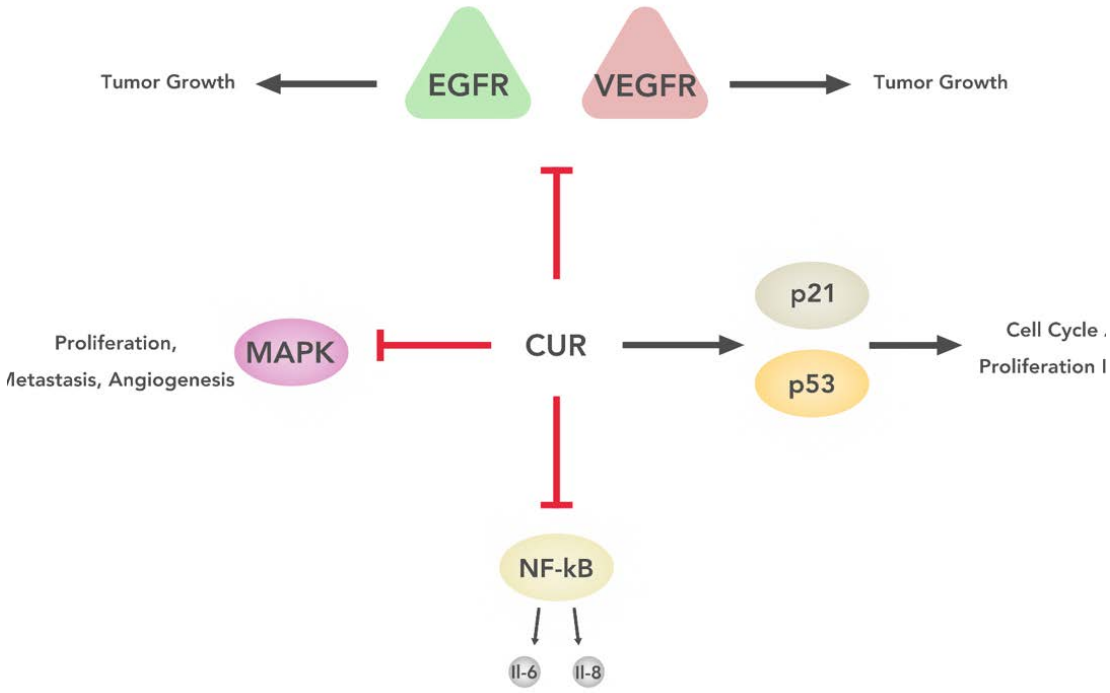

Figure 2-1: Curcumin acting on targets in HNSCC. Curcumin blocks EGFR, VEGFR, NF-κB and MAPK which suppresses tumor growth, cellular proliferation, metastasis, angiogenesis and cytokine Il-6 and Il-8. Curcumin promotes tumor suppressors p21 and p53 which promote normal cell cycle regulation and proliferation [66]

Arum palaestinum

Arum palaestinum is widely cultivated in Palestine, and has been used to treat cancer in Palestine for many years [71]. Extracts in ethanol of *Arum palaestinum* have demonstrated antitumor efficacy in prostate cancer, breast cancer, and leukemia [63, 72]. However, little has been done to elucidate *Arum palaestinum's* mechanism of action.

Figure 2-2: Nutraceuticals act on HNSCC Targets

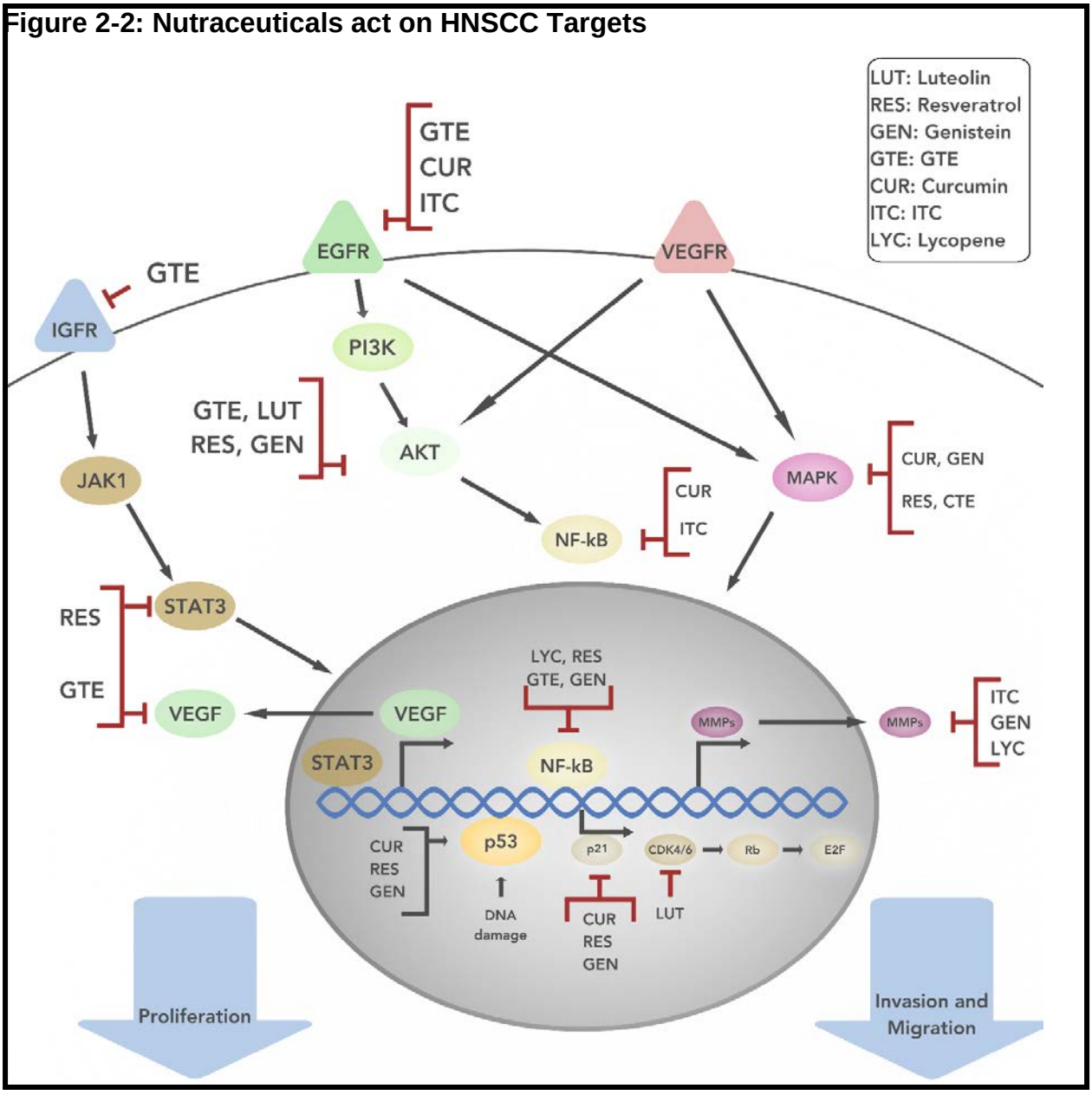

Figure 2-2: Multiple nutraceuticals act on tumor suppressors, transcription factors, and signaling molecules in head and neck cancer. [66]

Harmine

Peganum harmala contain alkaloids that are known to have a wide range of medicinal properties. The main component, harmine, demonstrates anti-tumor effects and inhibits monoamine oxidase [73]. Harmine has been shown to intercalate with DNA [74], and prevents chemotherapy resistance by interfering with drug efflux [75]. Harmine has little effect on normal cell lines while decreasing proliferation of various tumor lines [76].

While chemotherapies are often given to patients in combination, a systematic study of HNSCC response to nutraceuticals given in combination has yet to be investigated. The purpose of this study was to assess whether a potentiated or additive effect could be obtained by combining nutraceuticals. The previous work on curcumin in combination with cancer therapy [69] [70] was used as the starting point and included *Arum palaestinum* and *Peganum harmala* for their purported anti-tumor activity in HNSCC. The plant combination of dried extracts of three plants (GZ17-S), a synthetic variety of the extract (GZ17-5.0) and the three major anti-tumor synthetic compounds found in the original plants (GZ17-6.02) were assessed and compared. The results demonstrated a highly effective nutraceutical combination for treating HNSCC, and the proposed combination as more potent than their singular agents in preclinical models. The mechanism of action and evidence of a useful biomarker for future clinical study were delineated.

Chapter III: Methods

Sections of this chapter have been previously published as an open access article and are included here with adaptations with permission. Vishwakarma, V., et al., *Potent Antitumor Effects of a Combination of Three Nutraceutical Compounds.* Sci Rep, 2018. **8**(1): p. 12163.

Cells and Reagents

Well characterized HNSCC cell lines were used in this study[77]. HNSCC lines OSC19, HN5 and UM-SCC-1, Het1A a cancer-free, immortalized, esophageal line, glioblastoma line U87, and murine SCCvII/SF cultured in increased glucose DMEM (Corning, NY, USA) with 10% heat-inactivated fetal-bovine (FBS). Lung cancer lines A549 and 201T cultured in Hams F12K media (for A549) and Basal Eagle Media (for 201T) both augmented with 10% FBS. A maximum of 10-12 passages were used while growing all cell lines. HUVECs were cultured in endothelial basal medium-2 augmented with endothelial growth media-2 SingleQuot Kit (Lonza, Basel, Switzerland) using manufacturer's instructions. Primary HNSCC tumor tissue was collected through the Biospecimen Repository Core at the University of Kansas Cancer Center with written informed consent from patients, using protocols approved by the Institutional Review Boards at the University of Kansas Medical Center (KUMC).

The plant compound, GZ17-S, was provided by Genzada Pharmaceuticals (Sterling, KS) and prepared by combining curcumin, Arum Palaestinum, and Peganum harmala seeds, by means of a previously published protocol [78]. GZ17-5.00 formulation (Afaya Plus) was provided by Genzada Pharmaceuticals, and prepared by combining nutraceutical components: curcumin, piperonal, harmine (Indofine, Hillsborough, NJ), hydroxymethyl furfual, limonene, citraconic anhydride, benzyl nitrile, methylpyroglutamate, and isovanillin (all components from Sigma-Aldrich, St. Louis, MO). In the extraction procedure, linolenic acid (TCI Chemicals, Portland, OR), iodine solution (LabChem Inc, Zelienople, PA), diallyldisulfide (Sigma-Aldrich), and betasitosterol (MP Biomedicals, Santa Ana, CA) were used [79].

GZ17-6.02 was prepared by combining harmine, isovanillin, and curcumin (Sigma-Aldrich) (Supplemental Table 1). Cisplatin was obtained from Fresenius Kabi (purchased

by KUMC Pharmacy, Kansas City, KS). Tofacitinib was purchased from Selleckchem (Houston, TX). Recombinant human epidermal growth factor receptor (EGFR) was purchased from Fisher Scientific (Gibco, PHG0314, Waltham, MA) [79].

Cytotoxicity Assay

Cells ($4x10^3$ cells/well, 96-well plate) were treated in triplicate with GZ17-formulations at various concentrations, and cell proliferation assessed using CyQUANT assay kit (Life Technologies, Waltham, MA) using the manufacturer's instructions. After 72 h. ED_{50} calculated using non-linear curve fit with GraphPad Prism software version 6.03 (GraphPad Software Inc., San Diego, CA) [79].

HNSCC cells treated with cisplatin (4μM), GZ17-6.02 (ED_{50} of respective cell type), combination cisplatin and GZ17-6.02, or vehicle control for 72 h. Cell viability assessed using CyQUANT.

To assess the efficacy of GZ17-06.02 to potentiate the effects of radiation on HNSCC, OSC19 ($2x10^3$ cells/well in 96-well plates) were treated with 3, 6, and 9 Gy of radiation. Plates were exposed to gamma radiation (J.L. Shepherd and Associates Mark I Model 68A cesium-137 source irradiator; dose rate = 2.9 Gy/min). Media was aspirated, and cells were treated with GZ17-06.02 ED50 concentration or vehicle control in DMEM with 10% FBS [79].

Migration and invasion assay

Cells (2×10^4 cells per insert) were seeded in trans-well inserts with 8 μm pores (Becton Dickinson, Franklin Lakes, NJ). For invasion assay, a layer of diluted Matrigel (2 mg/ml) in DMEM (BD Biosciences, San Jose, CA) was placed in the insert. Cells in serum-free media were seeded on top of Matrigel layer for invasion or directly onto insert for migration assay. The inserts were placed in duplicate holding-wells containing GZ17-formulations (ED_{50}) in complete media for 24 h. Treated cells were plated in parallel to assess viability using CyQuant. The number of cells that moved to other side of membrane were counted after fixation and staining with Hema3 kit (Fisher Scientific). The numbers of invading or migrating cells were normalized to cell viability [79].

Tubule Formation Assay

HUVEC cell lines were plated on Matrigel in triplicate with GZ17-6.02 or vehicle control (15 x 10^3 cells/well of 96-well plate). After 6 hours, images were taken from 5 random fields per well. Images were analyzed using Pipeline software version 1.4 (Medical College of Wisconsin, Milwaukee, WI) according to published instructions to quantify total tube length [80].

Macrophage infiltration and viability

To test the effect of GZ17-formulations in attenuation of macrophage infiltration into the tumor, we used the metastatic mimetic device (MMD) as previously described [81]. 2.5 x 10^5 OSC19 cells were embedded in rat tail collagen in the MMD and allowed to gel overnight. 5x10^5 Thp1 were plated into the outer chamber of the device in serum containing medium. The collagen plugs were quantified using ImageJ software. Viability of Thp1 cells were assessed by counting cells on a hemocytometer using trypan blue dye exclusion. HNSCC cell viability was assessed by imaging cells stained with 0.1 µM Calcein AM dye for 60 min at 37°C in the dark [79].

Human phospho-kinase profiler array

Human phospho-kinase array (ARY003, Proteome Profiler™, R&D Systems, Minneapolis, MN) was used to identify signaling molecules regulated by GZ17-formulations per manufacturer's instructions. Membranes imaged by autoradiography, and ImageJ quantified signal intensity [79].

Immunoblotting

Cells (3 x 10^5 cells/60 mm dish) were treated with GZ17-formulations (ED_{50}) for 72 h and lysed in RIPA buffer containing protease/phosphatase inhibitors (cOmplete, Mini, Roche, Indianapolis, IN). Inhibition of EGF was stimulated via cell starvation for 48 hours, and then treated with GZ17-6.02 (ED_{50}) for 2 hours. EGF (10 ng/mL) was applied for 5 m, and then cells were harvested in RIPA buffer containing protease/phosphatase inhibitors on ice. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with p-ERK1/2 (p44/42-pMAPK; Thr202/Tyr204), total ERK1/2, cleaved PARP, caspase-3 (Cell Signaling, Danvers, MA) and β-tubulin (Fisher).

Immunoreactivity was detected using anti-mouse or anti-rabbit IgG conjugated to Dylight-680 or -800 (Fisher). Protein bands were detected using Li-Cor Odyssey protein imaging system (Li-Cor Biotechnology, Lincoln, NE) and quantified using ImageJ [79].

Computational Molecular Docking

Ligands designed using ACD/ChemSketch software (ACDlabs, Ontario, Canada). Crystal structures of proteins downloaded from RCSB-Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). AutoDock-Vina was employed for molecular docking using protein and ligand information. During the docking procedure, proteins were considered as rigid and ligands as flexible. The docking pose with lowest binding energy and highest binding affinity was aligned with the protein structure [79].

Cell cycle analysis

OSC-19 cells ($2x10^5$) were treated in triplicate with ED_{50} concentrations of GZ17-formulations for 72 h. Cells were washed with PBS, fixed with 70% ethanol, stained with propidium iodide (0.02mg/ml in PBS with 0.1% TritonX-100) (Life Technologies), and subjected to cell cycle analysis using Accuri C6 Flow Cytometer (BD Biosciences). Data were analyzed using the software provided by the manufacturer and samples analyzed in triplicate [79].

In vivo studies

Animal care was in strict compliance with IACUC guidelines at the University of Kansas Medical Center. Mice were housed in individually ventilated cages in a sterile environment, and all experimental work was conducted during daytime hours. A blind observer assessed all measured outcomes. To assess *in vivo* antitumor efficacy of GZ17-formulations, 1 x 10^6 HNSCC (OSC19) cells were injected subcutaneously into the flank of athymic nude-*Foxn1*^{nu} mice (Harlan Sprague Dawley, Indianapolis, IN). Tumor bearing mice were randomized when tumor volume reached 5.0 mm^3. Mice were treated with 15 mg/kg/day of GZ17-formulations or with saline control by 50 µL intratumoral administration once daily, five days/week. Tumor diameters were measured in two perpendicular dimensions and volume calculated as previously described, briefly (tumor

volume = long dimension x (short dimension)^2 x 0.52) [82]. Animals were euthanized by CO_2 asphyxiation followed by pneumothorax [79].

A syngeneic HNSCC model was used [83]. 1 x 10^6 SCC/vII cells were inoculated in the flank of C3H mice to assess GZ17-6.02 in immunocompetent hosts. Tumors were established for 6 days, and mice randomized into treatment groups (n=10/group). Treatment was delivered suspended in 1% carboxymethylcellulose (CMC) by oral gavage (100 mg/kg/day). Mouse weight and tumor volumes were measured three times per week [79].

To determine GZ17-6.02 efficacy in patient-derived HNSCC xenografts, 35 mg of primary HNSCC tissue was surgically implanted subcutaneously into flanks of athymic nude-*Foxn1*^{nu} mice under inhalant isofluorane anesthesia. Intraoperatively animals were administered analgesic Buprenorphine SR at 0.3 mg/kg and tofacitnib (to reduce natural killer cell counts) at 15 mg/kg via subcutaneous injections. Established tumors were passaged twice through mice before implantation for study. Tumor bearing mice were randomized in treatment groups (n=10/group). Mice were treated with 30 mg/kg GZ17-6.02 or 1% carboxymethycellulose control via oral gavage once daily, five days/week. On treatment day 8, the dose was increased to 50 mg/kg/day to improve antitumoral response. As PDX models have variable growth rates, data presented for *in vivo* studies as fractional tumor volume. Tumors were excised, snap frozen in dry ice and analyzed by immunoblotting [79].

Statistical Analysis

All results are cumulative from three independent experiments. For *in vitro* and *in vivo* experiments, unless otherwise indicated. Data analyzed using nonparametric Mann-Whitney tests. Analyses performed using Graphpad Prism Version 6.03.

Chapter IV: Results

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Combining curcumin, harmine, and isovanillin demonstrates potent cytotoxicity in multiple cell lines.

Varying concentrations of GZ17-formulations were tested on HNSCC cell lines (0, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 µg/mL) to determine the dose response to different formulations. GZ17-6.02 was the most cytotoxic (OSC19 cell ED50 = 11.85 µg/ml; UM-SCC-1 cell ED50 = 13.03 µg/ml; HN5 cell ED50 = 13.73 µg/ml) as compared to GZ17-5.00 and GZ17-S (Fig. 4-1 A). At 50 ug/mL no formula displayed complete cell killing, as such ED50 concentration was used for further studies. In Het1A cell line, a non-cancerous immortalized esophageal line, a higher ED50 was observed (Fig. 4-1B).

To eliminate the possibility these effects were anomalous to HNSCC cell lines, the proliferation of glioblastoma line U87, and lung cancer lines 201 T and A549 was assessed. GZ17-6.02 was cytotoxic in these cell lines congruent to the HNSCC lines tested (Fig. 4-1C). Thus, these results show that a combination of synthetic plant components decrease cancer cell proliferation.

Figure 4-1: GZ17 is cytotoxic to HNSCC and potentiates effects compared to single agents

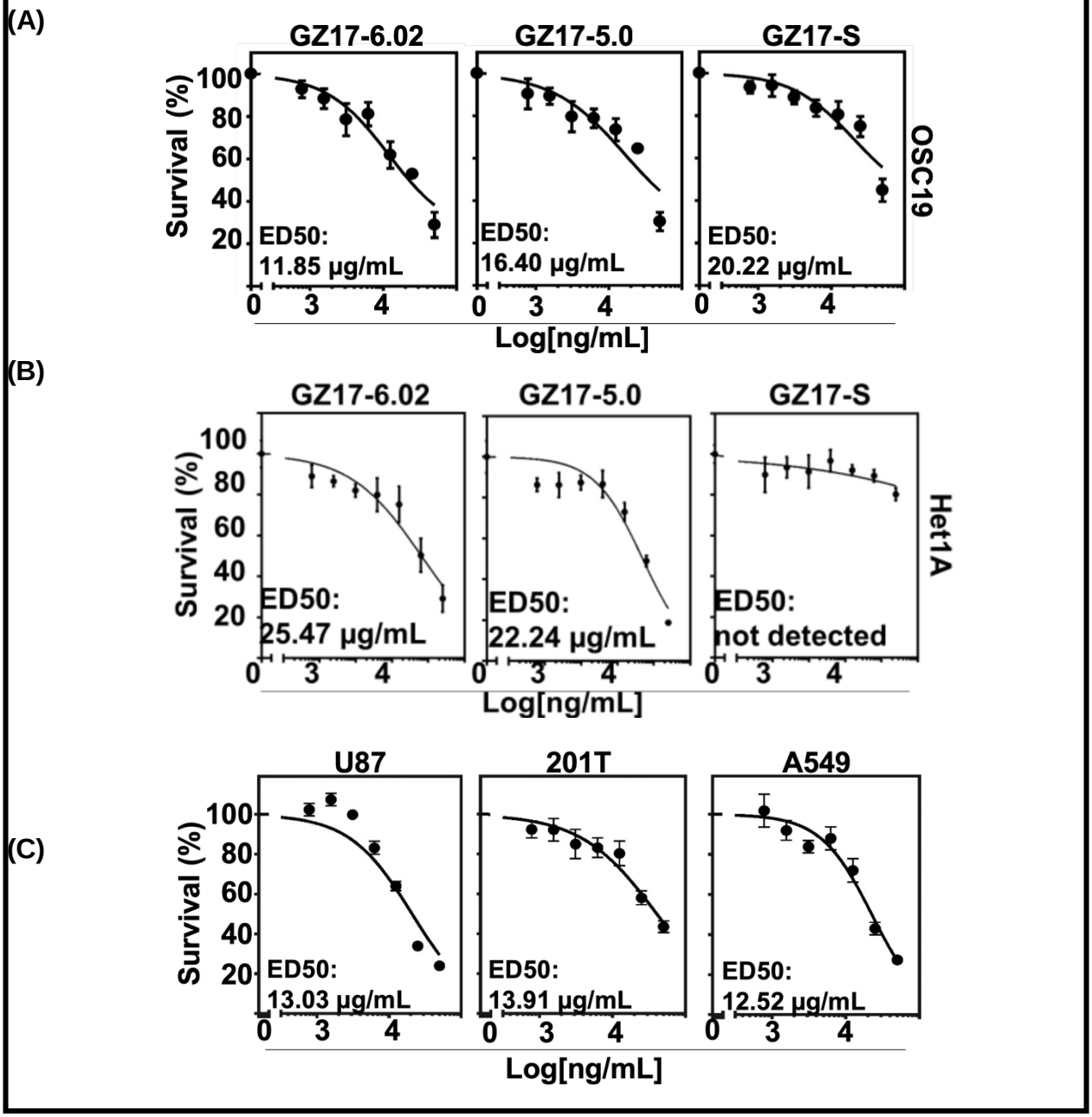

Figure 4-1: (A) OSC19 ($4x10^3$ cells/well in triplicate) were treated with different concentrations of GZ17-6.02, -5.0, and -S. Effective dose (ED^{50}) was calculated with non-linear curve fit using the software GraphPad Prism. Cumulative data signifies three separate experimental repeats and error bars represent ± SEM. **(B)** Het1A ($4x10^3$ cells/well in triplicate) were treated with different concentrations of GZ17-6.02, -5.0, and -S. Effective dose (ED^{50}) was calculated with non-linear curve fit using the software GraphPad Prism. Cumulative data signifies three separate experimental repeats and error bars represent ± SEM. **(**. **(C)** Glioblastoma (U87, and lung cancer lines (21T and A549) were treated with different concentrations of GZ17-6.02 to assess ED_{50} concentrations [79].

GZ17-6.02 and -05.00 induce apoptosis through PARP cleavage and caspase-3 activation.

HNSCC cells were treated for 72 hours at the ED_{50} dose. GZ17-05.00 and GZ17 induced significant apoptosis (24% and 22%, respectively, $P < 0.05$) demonstrated by the sub G0 fraction of cell cycle analysis in treated cells (Fig. 4-2 A). Poly-ADP ribose polymerase (PARP) and caspase – 3 were assessed using immunoblot. After GZ17 – 6.02 and GZ17 – 05.00 treatments at ED50 concentrations, HNSCC cells showed increase cleaved PARP and decreased caspase – 3 (Figure. 4-2 B), indicating treatment-induced apoptosis.

Figure 4-2: GZ17 treatments induce apoptosis via cleaved PARP cleavage and caspase-3 activation in HNSCC cells

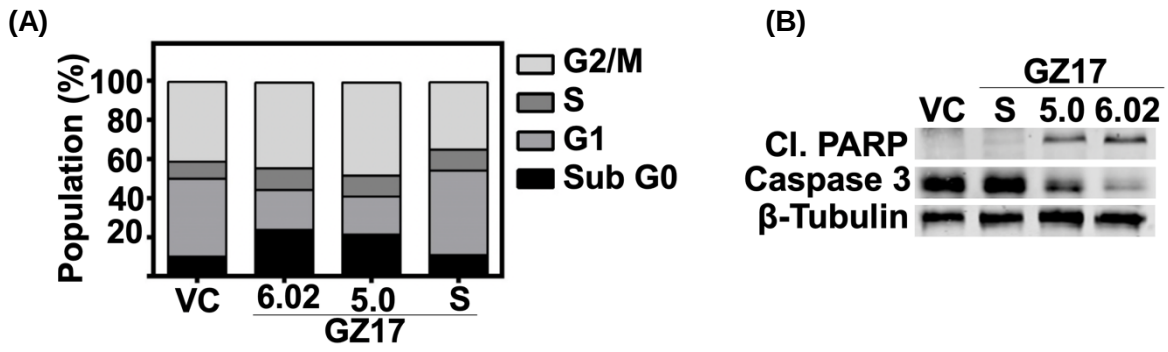

Figure: 4-2: (A) HNSCC cells (OSC19; 2 × 105 cells) were treated with vehicle control or ED_{50} concentrations of GZ17-6.02, -05.00 or –S for 72 h and analyzed via flow cytometry. The percentage of cells in different cell cycle stages is represented of each treatment of GZ17 formulation at ED_{50} concentration. The graph represents cumulative results from three independent experiments. (**B)** Representative immunoblot of apoptotic markers cleaved-PARP and caspase-3. β-tubulin levels show equal loading of protein across lanes [79].

GZ17-6.02 mitigates migration and invasion of HNSCC cells, and endothelial cell tubule formation.

The effects of GZ17-formulations on HNSCC migration and invasion were assessed. GZ17-6.02 (ED50 dose) was most effective in significantly inhibiting migration ($p < 0.0001$, Fig. 4-3A); and invasion ($p = 0.0003$, Fig. 4-3B). In addition, the inhibitory effects of GZ17-6.02 against glioblastoma and lung cancer cell line migration and invasion were assessed, and observed significant reductions in U87 (Fig. 4-3C), 201T (Fig. 4-3D), and A549 (Fig. 4-3E) migration and invasion.

Macrophage infiltration is often associated with an inflammatory reaction, and tumor-associated macrophages may facilitate tumor progression. A 3-dimensional cell culture device called the metastatic mimetic device (MMD) was used to assess macrophage infiltration into tumors when treated with GZ17-6.02 or vehicle control [84]. HNSCC cells embedded in a collagen matrix were subjected to macrophage infiltration in the MMD in the presence of vehicle control or GZ17-6.02. GZ17-6.02 effectively reduced macrophage survival (Fig. 4-3F) and subsequently infiltration through the collagen plugs. These functional assays demonstrate that GZ17- 6.02 significantly inhibited in vitro tumor progression.

Angiogenesis is the process through which HNSCC forms new blood vessels from pre-existing vessels, allowing access to nutrients required for the survival of tumor cells. However, no antiangiogenic therapies are approved for use in HNSCC28. We tested the efficacy of GZ17-6.02 (ED50 dose) to inhibit endothelial tubule formation, an in vitro indicator of angiogenesis [84]. GZ17-6.02 reduced tubule length relative to VC.

Figure 4-3: GZ17-6.02 effectively inhibits migration and invasion of HNSCC cells, and endothelial cell tubule formation

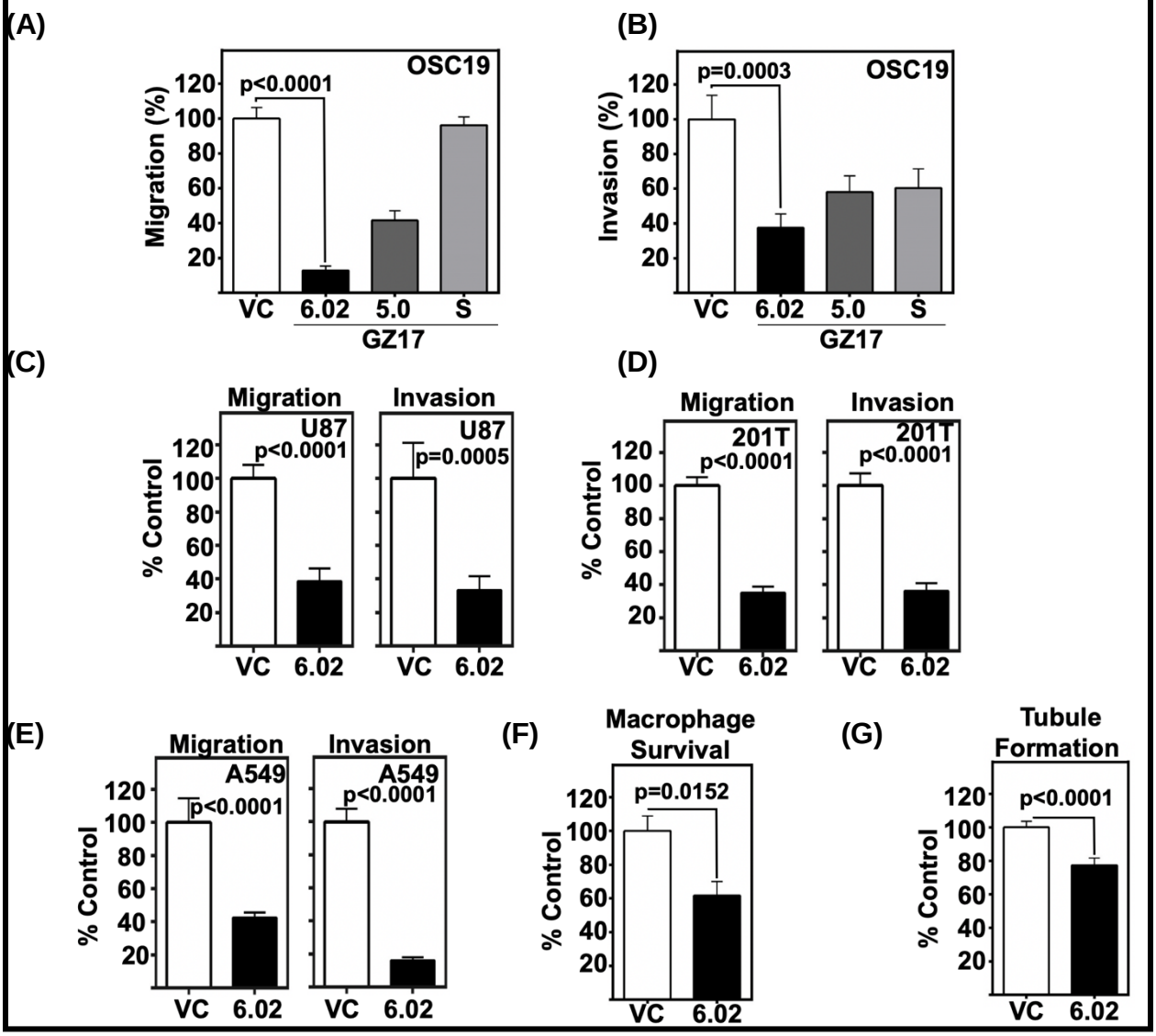

Figure 2. (**A, B)** HNSCC cells (OSC19; 2 × 103 cells/ well, plated in triplicate) were treated with ED50 concentrations of GZ17-6.02, -05.00 or -S, or vehicle control. Cell migration and invasion was determined at 24 h. The number of cells that (**A)** migrated or **(B)** invaded were counted and normalized to the cell viability. Percent migration or invasion compared to the vehicle control is shown in the graphs. Sum data represents three individual experimental repeats and error bars represent ± SEM. **(C,D,E)** Glioblastoma (U87), and **(D and E)** lung cancer (201T and A549) were determined for GZ17-6.02 inhibition of migration and invasion. Sum data represents three individual experimental repeats and error bars represent ± SEM. **(F)** GZ17- 6.02 inhibits tumor-promoting macrophage survival. Macrophage cell line Thp1 were treated with 60 µg/ml GZ17-6.02 for 48 h. Viable cells were counted using trypan blue dye exclusion. Graph represents sum results from five individual experiments and error bars represent ± SEM. **(G)** GZ17-6.02 modulates the angiogenic potential of HUVEC in vitro. HUVEC cells were treated with the ED50 dose (derived from OSC19) of GZ17- 6.02 and imaged 6 h after treatment and tubule formation was determined. Total tube length analyzed using Pipeline software from 15 random fields from each repeat, and normalized to vehicle control treated cells (VC). Sum data represents three individual experimental repeats and error bars represent ± SEM [79].

GZ17 -formulations modulate signaling molecules in HNSCC.

In vitro, GZ17 -formulations showed significant anti-cancer effects. This prompted a need to assess the mechanism of action and the additive effects observed. To test this, a phospho-protein array was employed to determine if signaling molecules were being modulated by GZ17 treatments. Phosphorylation of several signaling proteins including EGFR, and several downstream molecules including Src, ERK1/2, Akt, STAT-2, Chk-2 were significantly diminished in GZ17-treated HNSCC (ED_{50} dose) (Fig. 4-4 A, B). Other signaling pathways were represented in the array and some showed a modest decrease in phosphorylation (not shown). We focused on EGFR and its downstream molecules as these were most consistently and significantly decreased in the GZ17-6.02 treatment group. Further, EGFR is overexpressed and highly characterized in HNSCC; contributing to inhibition of apoptosis, activation of proliferation and angiogenesis, and radiotherapy resistance [85]. These signaling molecules are important modulators of HNSCC proliferation, migration, and survival.

ERK1/2 is a regulator of HNSCC proliferation, and increased levels are associated with advanced disease [86]. An immunoblot of pERK1/2 at both a short time interval when induced by EGF (5 min) and a long interval (72hr) validated the phosphor-protein array (Fig. 4-4 C). These data demonstrate that GZ17–6.02 inhibits the phosphorylation of ERK1/2, an important molecular mediator of HNSCC progression.

Figure 4-4: GZ17 formulations modulate key HNSCC signaling molecules

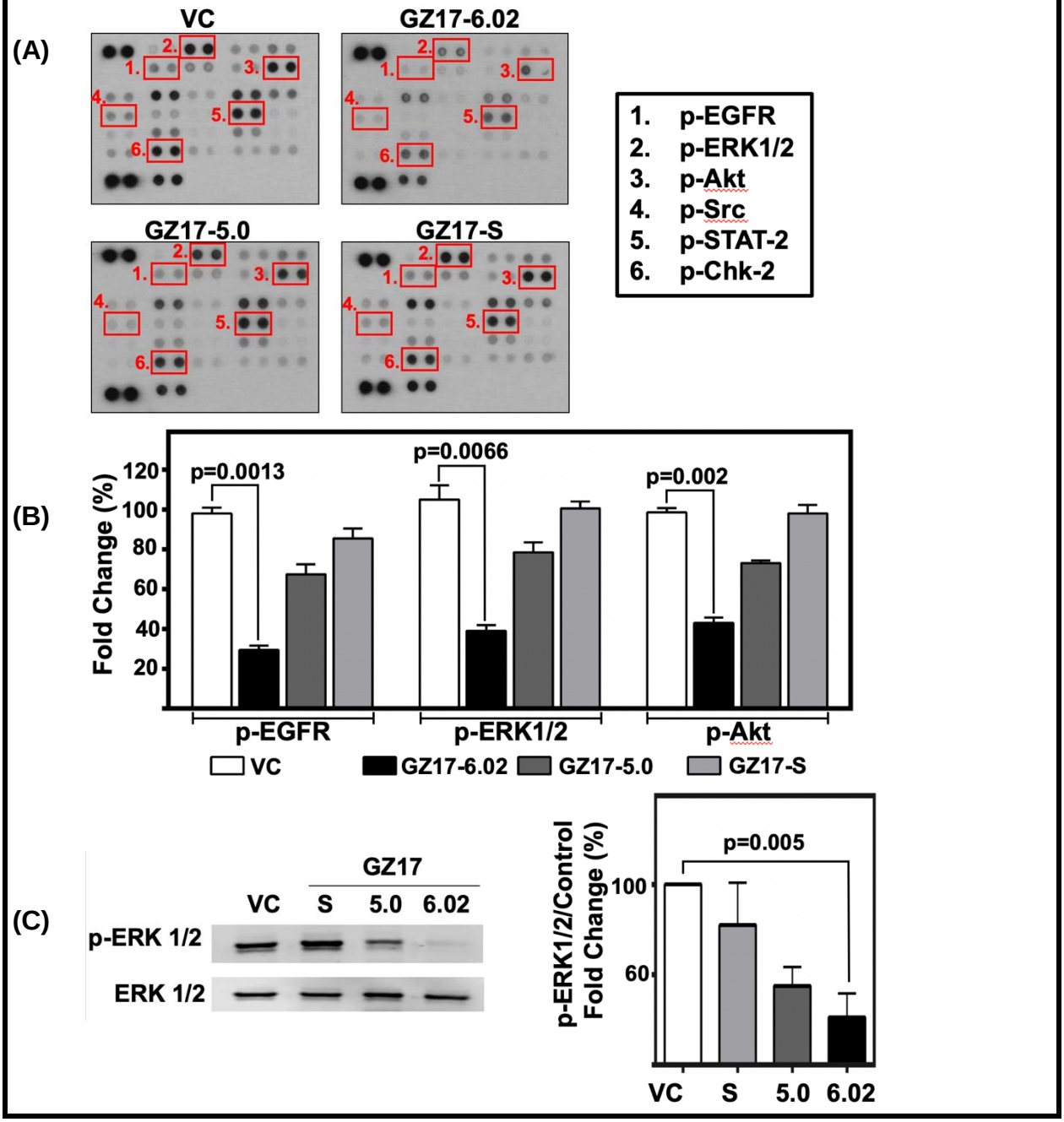

Figure 4-4. (A) HNSCC cells (OSC19; 2 × 105 cells) were treated with vehicle control or ED50 concentrations of GZ17-6.02, -05.00 or –S for 48 h. Representative dot-blot image from phospho-kinase array. **(B)** Densitometric analyses of dot-blot image signals from GZ17 treated lysates normalized to those from vehicle control and represented as fold change in protein levels. The graph shows sum data from two individual experiments. Error bars represent ± SEM. **(C)** HNSCC cells (OSC19; 2 × 105 cells) were treated with ED50 concentrations of GZ17-6.02, -05.00, –S or vehicle control for 72 h. Immunoblot was implemented for phospho-ERK1/2 and total ERK1/2 as loading control. Image is illustrative of three individual experimental repeats. Densitometric analyses of signals from immunoblots normalized to loading control and represented as fold change in protein levels relative to vehicle control treated cells. Error bars represent ± SEM [79].

GZ17-6.02 agents bind with affinity to EGFR, Akt1, and ERK1/2.

Docking analysis was performed to investigate the mechanisms of GZ17-6.02 on specific targets. Curcumin and harmine, along with their metabolites, displayed high binding affinity to several distinct binding sties on EGFR, Akt1, and ERK1-2 (Fig 4-5). Curcumin presented as the best binding agent at -6.6 Kcal/mol to Akt1 and -6.5 Kcal/mol to EGFR. Isovanillin did not show significant binding to the assessed targets. Simultaneous interaction to the target may explain the enhanced efficacy of the combination.

GZ17-6.02 enhances cisplatin efficacy.

The standard of care chemotherapy for HNSCC patients is platinum-based [36]. However, cisplatin resistant-tumors are a common occurrence in HNSCC therapy. HNSCC cell survival was tested against the combination of GZ17-6.02 (ED_{50}) with cisplatin (4.0 μM). The combination of cisplatin with GZ17-6.02 significantly reduced HNSCC cell survival as compared to either treatments alone ($p < 0.0008$, Fig. 4-6 A–C). A combination of radiation and GZ17-6.02 was found to have greater cytotoxicity than 3, 6, or 9 Gy of radiation alone (Fig. 4-6 D), with no additive benefit of radiation in combination. These data demonstrate GZ17-6.02 treatment potentiates the effects of standard chemotherapy commonly used in HNSCC protocol.

Figure 4-5: Curcumin and harmine bind with high affinity to distinct sites on ERK1, EGFR, and Akt-1

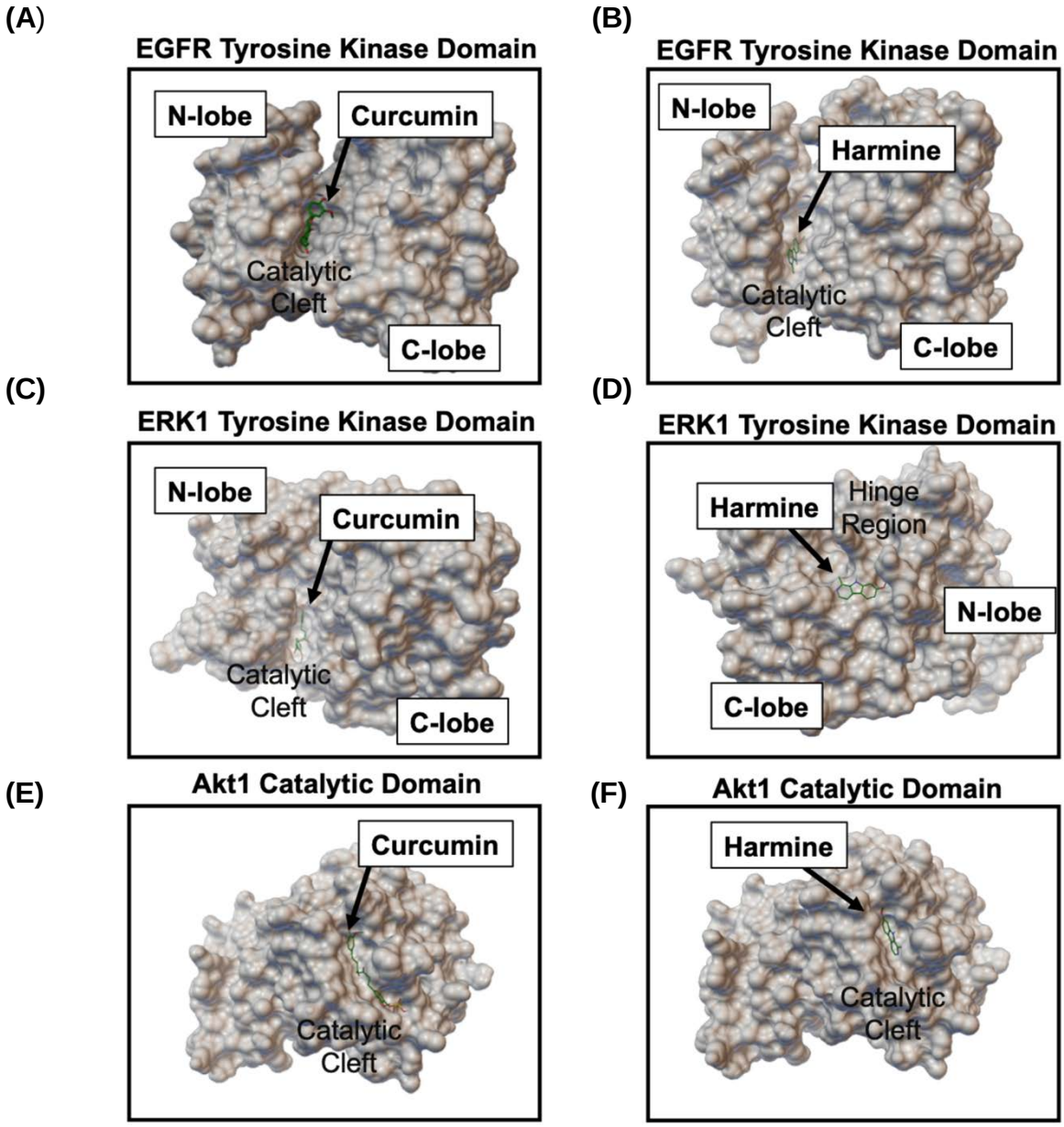

Figure 4-5. Binding conformation of top ranked docked poses of (A,C,E) curcumin and (B,D,F) harmine on EGFR tyrosine kinase domain, ERK1 tyrosine kinase domain and Akt1 catalytic domain, respectively [79].

Figure 4-6: The combination of GZ17-6.02 with cisplatin or radiation potentiates HNSCC cytotoxicity

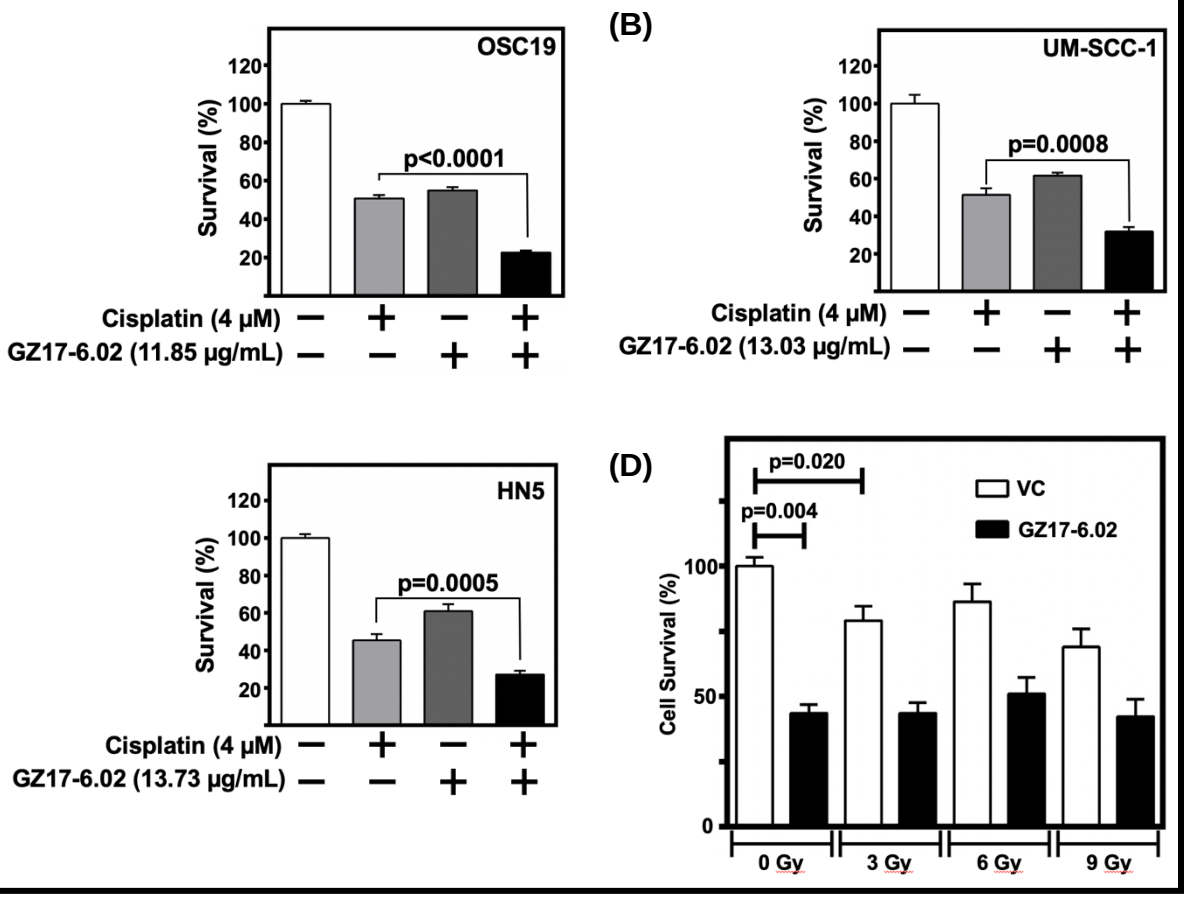

Figure 4-6: A) OSC19, (B) HN5, and (C) UM-SCC-1; (2 × 103 cells/well) in triplicate were treated with their respective ED50 concentrations of GZ17-6.02, cisplatin (4 μM), combination of both GZ17-6.02 and cisplatin, or vehicle control for 72 h. Cell survival was determined by CyQUANT assay. Obtained values were normalized to vehicle control and represented by fold change in cell survival. Sum data represents three individual experimental repeats plated in triplicate and error bars represent ± SEM [79].

GZ17-6.02 effectively inhibits HNSCC tumor growth *in vivo*.

GZ17 formulations had previously never been tested in animal models. To assess the efficacy of GZ17 *in vivo*, OSC19 tumors were injected in athymic nude mice with GZ17 formulations via intratumoral injection(15mg/kg/day) . Of all treatment groups, GZ17-6.02 was displayed the lowest increase in tumor volume ($p<0.001$, Fig. 4-7 A). A syngeneic SCC model was used to determine GZ17-6.02 in an immunocompetent host. SCCvII/SF syngeneic tumors are highly aggressive, requiring higher doses for testing. Therefore, GZ17-6.02 was given by oral gavage at a greater dose (100mg/kg/day). A significant decrease in tumor volume was seen by GZ17-6.02 treated mice ($p<0.001$, Fig 4-7 B). GZ17-6.02 treated mice demonstrated no abnormal behavior secondary to therapy; mice were active, curious, and behaved normally. Likewise, there was no difference in mouse weights between treatment groups (Fig. 4-7 C), and no adverse effects from treatment was evident. This shows GZ17-6.02 to be well tolerated in mouse models.

The response of GZ17-6.02 in patient-derived xenograft (PDX) tumors was assessed. GZ17-6.02 was given by oral gavage at a lower dose of 30 mg/kg/day for 8 days. The dose was then increased to 50mg/kg/day for the remaining duration of the study to improve the antitumoral response. GZ17-6.02 demonstrated significant anti-tumor effects compared to the vehicle control treated mice (<0.01, Fig. 4-7 D). Additionally, immunoblot was employed on GZ17-6.02 treated tumors to evaluate pERK1/2. Mice treated with GZ17-6.02 showed a significant decrease in pERK1/2 ($p=0.0006$ Fig. 4-7 E). GZ17-6.02 has demonstrated substantial anti-tumor effects in HNSCC preclinical models.

Figure 4-7: GZ17-6.02 demonstrates significant HNSCC antitumor effects *in vivo*

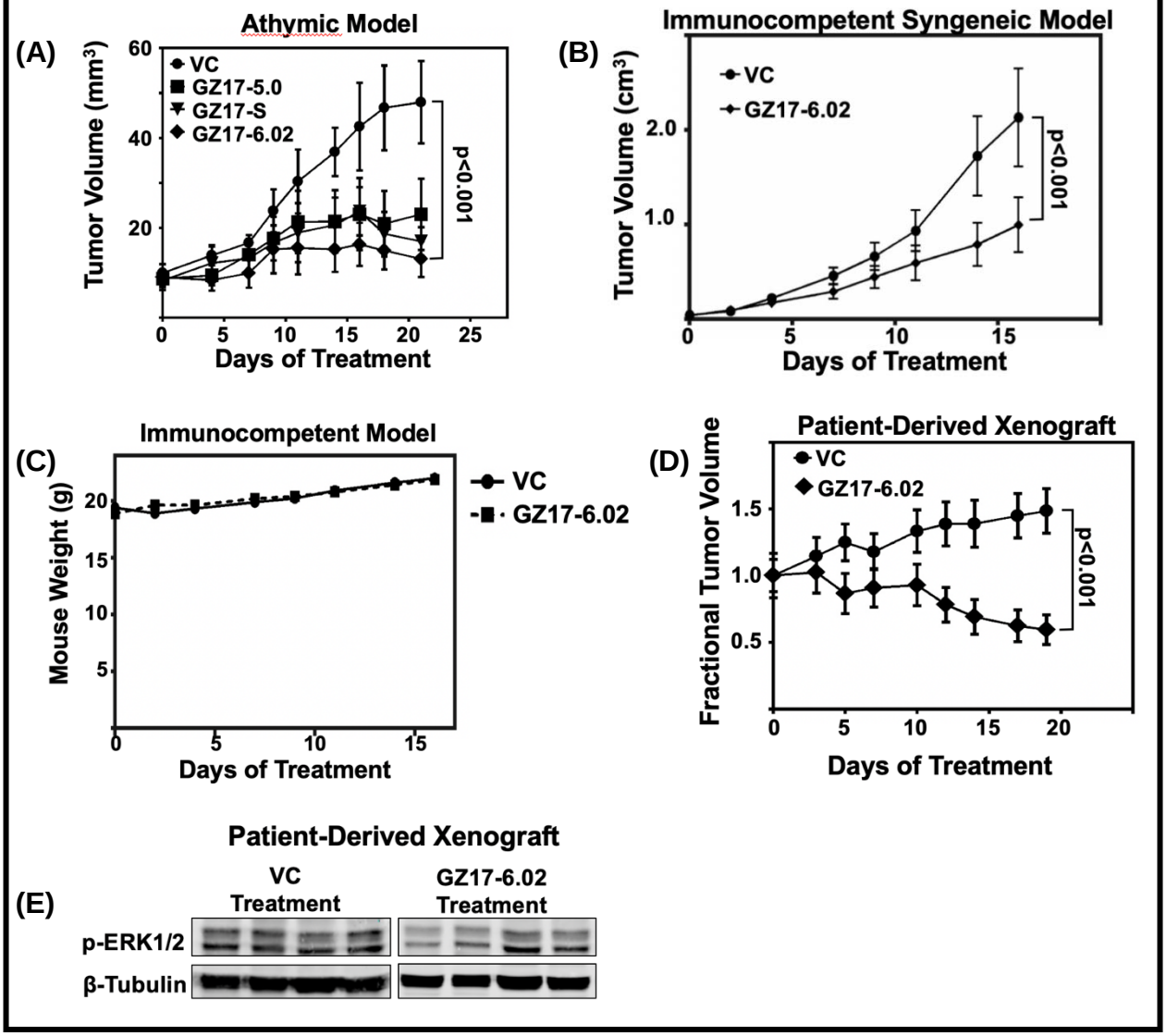

Figure 6. (A) HNSCC cells (OSC19; 1 × 106 cells) were injected subcutaneously on the flanks of athymic nude-Foxn1nu mice. Five mice per group were treated intratumorally with vehicle control (saline), or 15 mg/kg/d of GZ17-06.02, GZ17-5.0 or GZ17-S for three weeks. The graph shows tumor volumes measured with a vernier caliper over the course of the experiment. Error bars represent ± SEM. **(B)** Immunocompetent SCC/vII tumor bearing C3H mice were treated with GZ17-6.02 (100 mg/kg/day in 1% carboxymethylcellulose (CMC) suspension) or VC (1% CMC) by oral gavage (N = 10/group). The graph represents tumor volumes measured with a Vernier caliper over the course of the experiment. **(C)** Immunocompetent were administered via oral gavage either GZ17-6.02 (ED50) or vehicle control and weighed incrementally for 15 days. (**D)** Patient-derived HNSCC tumor masses (35 mg/site) were surgically implanted subcutaneously on both flanks of athymic nude-Foxn1nu mice. Ten mice per group were treated by oral gavage with GZ17-6.02 (30 mg/kg/d for first 7 days, and dose increased to 50 mg/kg/d to improve antitumoral effect) or vehicle control (saline) for 19 days. The graph represents fractional tumor volumes over the course of the experiment. **(E)** Representative immunoblot of patient-derived xenograft lysates demonstrates decrease in p-ERK1/2 levels. Densitometric analysis of p-ERK1/2 relative to density of loading control (β-tubulin) of GZ17-6.02 treated tumors (n = 8) compared relative to vehicle control (n = 8) were graphed. Error bars represent ± SEM.

Chapter V: Discussion

Sections of this chapter have been previously published as an open access article and are included here with adaptations with permission. Vishwakarma, V., et al., *Potent Antitumor Effects of a Combination of Three Nutraceutical Compounds.* Sci Rep, 2018. **8**(1): p. 12163.

Few studies have studied the combination of synthetic plant compounds to potentiate their effects [69], and no studies have previously been published examining a synthetic plant compound combination therapy for HNSCC. A combination therapy of known anti-cancer synthetic plant compounds could have additive antitumor effects. However, it is previously known that overly complex combinations of natural compounds may actually worsen the expected antitumor effects [87]. In this work, the plant compound curcumin, which is previously shown to have efficacy in combination formulations, was combined with two other plant compounds indicated to have anti-tumor activity, *Arum palaestinum* and *Peganum harmala.* The results obtained in this work rationalize the combination of GZ17-6.02, a mix of isovanillin, harmine, and curcumin as a more potent combination than single agents when evaluating anti-tumor efficacy. This, in tandem with a recent boon in plant derived compound research showing efficacy and safety[88], endorses GZ17-6.02 for further investigation.

GZ17-6.02 induced significant apoptosis via caspase-3, and inhibited phosphorylation of ERK1/2. This verifies previous findings that curcumin benefits cisplatin HNSCC treatment efficacy by increasing caspase 3/9 activity [89], and in breast cancer curcumin reduces proliferation by inhibiting phosphorylation of ERK1/2. Likewise, curcumin demonstrates improved effects when in combination with other

natural compounds or known drugs in other cancer types [69]. The production of reactive oxygen species is important to the development of macrophage-mediated inflammation-induced tumor progression. Importantly, curcumin is known to reduce the production of reactive oxygen species in macrophages [90]. Additionally, curcumin has been demonstrated to stimulate macrophages to the tumor inhibitory M1 phenotype [91]. The MMD model revealed significant reduction in macrophage volume when treated with GZ17-6.02. GZ17-6.02 demonstrated the ability to reduce macrophages viability and infiltration into tumor *in vitro*. GZ17-6.02 was also shown to reduce HNSCC proliferation and angiogenesis. This work corroborates previous work that demonstrated β-carboline (naturally occurring in harmine) to have anti-tumor and anti-angiogenic effects via intercalation of DNA, damaging it, and inducing tumor-suppressor p53 [92, 93]. Isovanillin has been proposed as a potential antitumor compound [72], and is known for its antioxidant effects

Cancer is vastly complex and presents with many different oncogenic pathways. This makes the ability of a drug to possess multiple targets highly desirable [94]. GZ17-6.02 demonstrated this multi-target ability by reducing Akt1, EGFR, and ERK1/2 and has high binding affinity to these targets. Additionally, Akt1 and EGFR mutations can induce chemotherapy resistance [95, 96]. As cisplatin resistance is a common occurrence in HNSCC treatment, it would be advantageous for a compound to target multiple molecules in a signaling pathway potentially relieving cisplatin resistance. This work reveals GZ17-6.02 to potentiate the effects of cisplatin. Feng et al. have previously shown that inhibition of Chk-2 enhanced the sensitivity of HNSCC to radiotherapy and

cisplatin [97]. This work shows that GZ17-6.02 deactivated Chk-2, which may explain the potentiated effect of this compound with cisplatin.

Many plant derived compounds are commonly limited by poor bioavailability. GZ17-6.02 seems to have overcome this. *In vivo*, GZ17-6.02 decreased HNSCC xenograft tumors, syngeneic SCC tumors in an immunocompetent mouse model, and patient-derived xenografts of HNSCC tumors. The initial dose of GZ17-6.02 was 15mg/kg/day via intratumoral injection was increased to 100mg/kg/day in the syngeneic model to establish *in vivo* dose. In the PDX model, 30mg/kg/day of GZ17-6.02 was administered via oral gavage. However, to improve tumor response the dose was increased to 50mg/kg/day on the eighth day. The mice tolerated all GZ17 formulations well, exhibiting no abnormal behavior, and maintaining body weight between different treatments of GZ17. Furthermore, *in vitro* analysis of non-cancerous Het1A cell line displayed a higher ED50 compared to cancer lines tested.

GZ17-6.02 displayed significant potentiated effects of standard of care therapy in both *in vitro* and *in vivo* animal models. *In vivo* phospho-ERK1/2 immunoblot revealed that GZ17-6.02 reduced p-ERK1/2 levels, supporting the *in vitro* model of phosphor-ERK1/2 reduction and docking analysis of ERK1/2.

Limitations

No testing of separate components of GZ17-6.02 were tested *in vivo*. However, the single components alone were not significantly potent compared to GZ17-6.02 *in vitro*. Further, there is little clinical efficacy of the single compounds. There were no observed toxicities to GZ17-6.02 in the *in vivo* models tested.

Therefore, the evidence presented proposes GZ17-6.02 to possess anti-tumor effects in HNSCC in preclinical models, and lay the foundation for future clinical trials using this treatment.

Supplementary Table 1

Supplemental Table 1: Synthetic components in GZ17-0.5.00 and GZ17-0.6.02

GZ17-05.00	
Component	*µg/mL of water*
Isovanillin	5.882
Beta-sitosterol	11.765
Linolenic Acid	4.059
Harmine	9.941
Hydroxymethyl furfural	5.529
Piperonal	2.000
Citraconic anhydride	2.235
Methylpyroglutamate	2.529
Curcumin	7.529
Benzyl nitrile	4.412
Limonene	2.941
GZ17-06.02	
Component	*mg/mL of ethanol*
Isovanillin	771
Harmine	130.3
Curcumin	98.7

References

1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA: A Cancer Journal for Clinicians, 2015. **65**(2): p. 87-108.
2. Braakhuis, B.J., C.R. Leemans, and O. Visser, *Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011.* Oral Oncol, 2014. **50**(7): p. 670-5.
3. Peng, K.A., T. Grogan, and M.B. Wang, *Head and neck sarcomas: analysis of the SEER database.* Otolaryngol Head Neck Surg, 2014. **151**(4): p. 627-33.
4. Pellitteri, P.K., et al., *Paragangliomas of the head and neck.* Oral Oncology, 2004. **40**(6): p. 563-575.
5. Carey, R.M., et al., *Patient, disease, and treatment factors associated with overall survival in esthesioneuroblastoma.* Int Forum Allergy Rhinol, 2017. **7**(12): p. 1186-1194.
6. Kalinowski, A. and K. Humphreys, *Governmental standard drink definitions and low-risk alcohol consumption guidelines in 37 countries.* Addiction, 2016. **111**(7): p. 1293-1298.
7. Maasland, D.H., et al., *Alcohol consumption, cigarette smoking and the risk of subtypes of head-neck cancer: results from the Netherlands Cohort Study.* BMC Cancer, 2014. **14**: p. 187.
8. Chaturvedi, A.K., et al., *Human Papillomavirus and Rising Oropharyngeal Cancer Incidence in the United States.* Journal of Clinical Oncology, 2011. **29**(32): p. 4294-4301.
9. Gillison, M.L., et al., *Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers.* Journal of the National Cancer Institute, 2008. **100**(6): p. 407-420.
10. Vidal, L. and M.L. Gillison, *Human papillomavirus in HNSCC: recognition of a distinct disease type.* Hematology/oncology clinics of North America, 2008. **22**(6): p. 1125-vii.
11. Tanaka, T.I. and F. Alawi, *Human Papillomavirus and Oropharyngeal Cancer.* Dent Clin North Am, 2018. **62**(1): p. 111-120.
12. Gillison, M.L., et al., *Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2015. **33**(29): p. 3235-3242.
13. Jit, M., et al., *Cost-effectiveness of female human papillomavirus vaccination in 179 countries: a PRIME modelling study.* Lancet Glob Health, 2014. **2**(7): p. e406-14.
14. Goldenberg, D.D., et al., *Habitual Risk Factors for Head and Neck Cancer.* Otolaryngology-Head and Neck Surgery, 2004. **131**(6): p. 986-993.
15. Wheeler, S., et al., *Enhancement of head and neck squamous cell carcinoma proliferation, invasion, and metastasis by tumor-associated fibroblasts in preclinical models.* Head Neck, 2014. **36**(3): p. 385-92.
16. Marur, S. and A.A. Forastiere, *Head and Neck Squamous Cell Carcinoma: Update on Epidemiology, Diagnosis, and Treatment.* Mayo Clinic Proceedings, 2016. **91**(3): p. 386-396.
17. Chaturvedi, A.K., et al., *Human papillomavirus and rising oropharyngeal cancer incidence in the United States.* J Clin Oncol, 2011. **29**(32): p. 4294-301.
18. D'Souza, G., et al., *Case-control study of human papillomavirus and oropharyngeal cancer.* N Engl J Med, 2007. **356**(19): p. 1944-56.
19. Insalaco, L., *Head and Neck Squamous Cell Carcinoma*, in *Ferri's Clinical Advisor*, F.F. Ferri, Editor. 2017, Elsevier, Inc.: Philadelphia, PA. p. 541-542.

20. Fakhry, C., et al., *The prognostic role of sex, race, and human papillomavirus in oropharyngeal and nonoropharyngeal head and neck squamous cell cancer.* Cancer, 2017. **123**(9): p. 1566-1575.
21. Lydiatt, W.M., et al., *Head and Neck cancers—major changes in the American Joint Committee on cancer eighth edition cancer staging manual.* CA: A Cancer Journal for Clinicians, 2017. **67**(2): p. 122-137.
22. Warnakulasuriya, S. and A. Ariyawardana, *Malignant transformation of oral leukoplakia: a systematic review of observational studies.* J Oral Pathol Med, 2016. **45**(3): p. 155-66.
23. Vermorken, J.B. and P. Specenier, *Optimal treatment for recurrent/metastatic head and neck cancer.* Annals of Oncology, 2010. **21**(suppl_7): p. vii252-vii261.
24. Slaughter, D.P., H.W. Southwick, and W. Smejkal, *"Field cancerization" in oral stratified squamous epithelium. Clinical implications of multicentric origin.* Cancer, 1953. **6**(5): p. 963-968.
25. Tabor, M.P., et al., *Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications.* Clin Cancer Res, 2001. **7**(6): p. 1523-32.
26. Lorenz, R., M. Couch, and B. Burkey, *Head and Neck*, in *Sabiston Textbook of Surgery*, J.D.B. Courtney Townsend, Mark Evers, Kenneth Mattox, Editor. 2017, Elsevier: Philadelphia, PA. p. 788-818.
27. Barcellos-Hoff, M.H., C. Park, and E.G. Wright, *Radiation and the microenvironment - tumorigenesis and therapy.* Nat Rev Cancer, 2005. **5**(11): p. 867-875.
28. Corvò, R., *Evidence-based radiation oncology in head and neck squamous cell carcinoma.* Radiotherapy and Oncology, 2007. **85**(1): p. 156-170.
29. Straub, J.M., et al., *Radiation-induced fibrosis: mechanisms and implications for therapy.* J Cancer Res Clin Oncol, 2015. **141**(11): p. 1985-1994.
30. Pitman, S.W., D. Miller, and R. Weichselbaum, *Initial adjuvant therapy in advanced squamous cell carcinoma of the head and neck employing weekly high dose methotrexate with leucovorin rescue.* Laryngoscope, 1978. **88**(4): p. 632-8.
31. Hong, W.K. and R. Bromer, *Chemotherapy in head and neck cancer.* N Engl J Med, 1983. **308**(2): p. 75-9.
32. Grose, W.E., et al., *Comparison of methotrexate and cisplatin for patients with advanced squamous cell carcinoma of the head and neck region: a Southwest Oncology Group Study.* Cancer Treat Rep, 1985. **69**(6): p. 577-81.
33. Clavel, M., et al., *Combination chemotherapy with methotrexate, bleomycin, and vincristine with or without cisplatin in advanced squamous cell carcinoma of the head and neck.* Cancer, 1987. **60**(6): p. 1173-7.
34. Morton, R.P., et al., *Cisplatinum and bleomycin for advanced or recurrent squamous cell carcinoma of the head and neck: a randomised factorial phase III controlled trial.* Cancer Chemother Pharmacol, 1985. **15**(3): p. 283-9.
35. Sacco, A.G. and E.E. Cohen, *Current Treatment Options for Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma.* Journal of Clinical Oncology, 2015. **33**(29): p. 3305-3313.
36. Vermorken , J.B., et al., *Platinum-Based Chemotherapy plus Cetuximab in Head and Neck Cancer.* New England Journal of Medicine, 2008. **359**(11): p. 1116-1127.
37. Ferris, R., *Immunology and Immunotherapy of Head and Neck Cancer.* Journal of Clinical Oncology, 2015. **33**(29): p. 3293-3304.
38. Schildhaus, H.U., *[Predictive value of PD-L1 diagnostics].* Pathologe, 2018. **39**(6): p. 498-519.
39. Ferris, R.L., et al., *Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck.* New England Journal of Medicine, 2016. **375**(19): p. 1856-1867.
40. Seiwert, T.Y., et al., *Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial.* The Lancet Oncology. **17**(7): p. 956-965.

41. Kelly, K., *The History of Medicine: Early Civilizations, Prehistoric Times to 500 C.E.* 2009, New York, New York: Facts on File.
42. Guglani, L. and C.L. Joseph, *Asthma and diet: could food be thy medicine?* Indian pediatrics, 2015. **52**(1): p. 21-22.
43. Flint-Hamilton, K.B., *Legumes in Ancient Greece and Rome: Food, Medicine, or Poison?* Hesperia: The Journal of the American School of Classical Studies at Athens, 1999. **68**(3): p. 371-385.
44. Jackson, W.A., *A short guide to humoral medicine.* Trends in Pharmacological Sciences, 2001. **22**(9): p. 487-489.
45. Jouanna, J., *Greek Medicine from Hippocrates to Galen : Selected Papers*. 2012, PB - Brill.
46. Dear, P., *Revolutionizing the Sciences European Knowledge and Its Ambitions, 1500-1700 - Second Edition*. 2009, Princeton, New Jersey: Princeton University Press. 216.
47. Harvey, W., *The anatomical exercises : De motu cordis and De circulatione sanguinis, in English translation*. 1995: Dover edition. New York : Dover Publications, 1995.
48. Virchow, R., *Cellular Pathology (1858)*. 2004: Thoemmes Continuum.
49. Goldacre, B., *Bad science: quacks, hacks, and big pharma flacks*. 2011, Toronto: Emblem.
50. Schultz, J., *The Discovery of Vitamin C by Albert Szent-Gyorgi.* American Chemical Society, 2002.
51. Brower, V., *Back to nature: extinction of medicinal plants threatens drug discovery.* J Natl Cancer Inst, 2008. **100**(12): p. 838-9.
52. Johnson, I.S., et al., *THE VINCA ALKALOIDS: A NEW CLASS OF ONCOLYTIC AGENTS.* Cancer Res, 1963. **23**: p. 1390-427.
53. Andlauer, W. and P. Fürst, *Nutraceuticals: a piece of history, present status and outlook.* Food Research International, 2002. **35**(2): p. 171-176.
54. Kalra, E.K., *Nutraceutical--definition and introduction.* AAPS pharmSci, 2003. **5**(3): p. E25-E25.
55. Alhasan, S.A., O. Aranha, and F.H. Sarkar, *Genistein elicits pleiotropic molecular effects on head and neck cancer cells.* Clin Cancer Res, 2001. **7**(12): p. 4174-81.
56. Lin, H.-Y., et al., *Resveratrol causes COX-2- and p53-dependent apoptosis in head and neck squamous cell cancer cells.* Journal of Cellular Biochemistry, 2008. **104**(6): p. 2131-2142.
57. Zhang, H., et al., *Luteolin induces apoptosis by activating Fas signaling pathway at the receptor level in laryngeal squamous cell line Hep-2 cells.* European Archives of Oto-Rhino-Laryngology, 2014. **271**(6): p. 1653-1659.
58. Yeh, Y.-T., et al., *Phenethyl isothiocyanate induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations.* Free Radical Biology and Medicine, 2014. **74**: p. 1-13.
59. Singh, M., et al., *Efficacy of oral lycopene in the treatment of oral leukoplakia.* Oral Oncology, 2004. **40**(6): p. 591-596.
60. Papadimitrakopoulou, V.A., et al., *Randomized Trial of 13-cis Retinoic Acid Compared With Retinyl Palmitate With or Without Beta-Carotene in Oral Premalignancy.* Journal of Clinical Oncology, 2009. **27**(4): p. 599-604.
61. Kim, S.G., et al., *Curcumin Treatment Suppresses IKKβ Kinase Activity of Salivary Cells of Patients with Head and Neck Cancer: A Pilot Study.* Clinical Cancer Research, 2011. **17**(18): p. 5953-5961.
62. Saba, N.F., et al., *Prevention of head and neck squamous cell carcinoma: Removing the "chemo" from "chemoprevention".* Oral Oncology, 2015. **51**(2): p. 112-118.
63. Husein, A.I., et al., *In vitro antioxidant and antitumor activities of six selected plants used in the Traditional Arabic Palestinian herbal medicine.* Pharmaceutical Biology, 2014. **52**(10): p. 1249-1255.
64. Notarbartolo, M., et al., *Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB activation levels and in IAP gene expression.* Cancer Letters, 2005. **224**(1): p. 53-65.

65. Lin, S.-S., et al., *Curcumin inhibits the migration and invasion of human A549 lung cancer cells through the inhibition of matrix metalloproteinase-2 and -9 and Vascular Endothelial Growth Factor (VEGF).* Cancer Letters, 2009. **285**(2): p. 127-133.
66. Crooker, K., et al., *A Review of Promising Natural Chemopreventive Agents for Head and Neck Cancer.* Cancer Prev Res (Phila), 2018. **11**(8): p. 441-450.
67. Cheng, A.L., et al., *Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions.* Anticancer Res, 2001. **21**(4b): p. 2895-900.
68. Tamvakopoulos, C., et al., *Metabolism and Anticancer Activity of the Curcumin Analogue, Dimethoxycurcumin.* Clinical Cancer Research, 2007. **13**(4): p. 1269-1277.
69. Saldanha, S.N. and T.O. Tollefsbol, *The role of nutraceuticals in chemoprevention and chemotherapy and their clinical outcomes.* J Oncol, 2012. **2012**: p. 192464.
70. Kunnumakkara, A.B., et al., *Curcumin Potentiates Antitumor Activity of Gemcitabine in an Orthotopic Model of Pancreatic Cancer through Suppression of Proliferation, Angiogenesis, and Inhibition of Nuclear Factor-κB–Regulated Gene Products.* Cancer Research, 2007. **67**(8): p. 3853-3861.
71. Ali-Shtayeh, M.S., et al., *Traditional knowledge of wild edible plants used in Palestine (Northern West Bank): A comparative study.* Journal of Ethnobiology and Ethnomedicine, 2008. **4**(1): p. 13.
72. Cole, C., et al., *Arum Palaestinum with isovanillin, linolenic acid and β-sitosterol inhibits prostate cancer spheroids and reduces the growth rate of prostate tumors in mice.* BMC Complementary and Alternative Medicine, 2015. **15**(1): p. 264.
73. Patel, K., et al., *A review on medicinal importance, pharmacological activity and bioanalytical aspects of beta-carboline alkaloid "Harmine".* Asian Pacific Journal of Tropical Biomedicine, 2012. **2**(8): p. 660-664.
74. Boeira, J.M., et al., *Genotoxic and recombinogenic activities of the two beta-carboline alkaloids harman and harmine in Saccharomyces cerevisiae.* Mutat Res, 2002. **500**(1-2): p. 39-48.
75. Ma, Y. and M. Wink, *The beta-carboline alkaloid harmine inhibits BCRP and can reverse resistance to the anticancer drugs mitoxantrone and camptothecin in breast cancer cells.* Phytotherapy Research, 2010. **24**(1): p. 146-149.
76. Song, Y., et al., *Specific inhibition of cyclin-dependent kinases and cell proliferation by harmine.* Biochemical and Biophysical Research Communications, 2004. **317**(1): p. 128-132.
77. Lin, C.J., et al., *Head and neck squamous cell carcinoma cell lines: established models and rationale for selection.* Head Neck, 2007. **29**(2): p. 163-88.
78. Suh, Y., et al., *Clinical update on cancer: molecular oncology of head and neck cancer.* Cell Death & Disease, 2014. **5**(1): p. e1018.
79. Vishwakarma, V., et al., *Potent Antitumor Effects of a Combination of Three Nutraceutical Compounds.* Sci Rep, 2018. **8**(1): p. 12163.
80. Yeh, Y.T., et al., *Phenethyl isothiocyanate induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations.* Free Radic Biol Med, 2014. **74**: p. 1-13.
81. Zhang, H., et al., *Luteolin induces apoptosis by activating Fas signaling pathway at the receptor level in laryngeal squamous cell line Hep-2 cells.* Eur Arch Otorhinolaryngol, 2014. **271**(6): p. 1653-9.
82. Wheeler, S.E., et al., *Enhancement of head and neck squamous cell carcinoma proliferation, invasion, and metastasis by tumor-associated fibroblasts in preclinical models.* Head Neck, 2013.
83. Smith, L.P. and G.R. Thomas, *Animal models for the study of squamous cell carcinoma of the upper aerodigestive tract: A historical perspective with review of their utility and limitations. Part A. Chemically-induced de novo cancer, syngeneic animal models of HNSCC, animal models of*

transplanted xenogeneic human tumors. International Journal of Cancer, 2006. **118**(9): p. 2111-2122.
84. Arnaoutova, I. and H.K. Kleinman, *In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract.* Nature Protocols, 2010. **5**: p. 628.
85. Arnold, L., J. Enders, and S.M. Thomas, *Activated HGF-c-Met Axis in Head and Neck Cancer.* Cancers (Basel), 2017. **9**(12).
86. Albanell, J., et al., *Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments.* Cancer Res, 2001. **61**(17): p. 6500-10.
87. Spindler, S.R., P.L. Mote, and J.M. Flegal, *Lifespan effects of simple and complex nutraceutical combinations fed isocalorically to mice.* AGE, 2014. **36**(2): p. 705-718.
88. Gupta, S.C., et al., *Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals.* Cancer and Metastasis Reviews, 2010. **29**(3): p. 405-434.
89. Duarte, V.M., et al., *Curcumin Enhances the Effect of Cisplatin in Suppression of Head and Neck Squamous Cell Carcinoma via Inhibition of IKKβ Protein of the NFκB Pathway.* Molecular Cancer Therapeutics, 2010. **9**(10): p. 2665-2675.
90. Joe, B. and B.R. Lokesh, *Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages.* Biochim Biophys Acta, 1994. **1224**(2): p. 255-63.
91. Tu, S.P., et al., *Curcumin Induces the Differentiation of Myeloid-Derived Suppressor Cells and Inhibits Their Interaction with Cancer Cells and Related Tumor Growth.* Cancer Prevention Research, 2012. **5**(2): p. 205-215.
92. Dai, F., et al., *A natural small molecule harmine inhibits angiogenesis and suppresses tumour growth through activation of p53 in endothelial cells.* PLoS One, 2012. **7**(12): p. e52162.
93. Hamsa, T.P. and G. Kuttan, *Harmine inhibits tumour specific neo-vessel formation by regulating VEGF, MMP, TIMP and pro-inflammatory mediators both in vivo and in vitro.* European Journal of Pharmacology, 2010. **649**(1): p. 64-73.
94. Jänne, P.A., N. Gray, and J. Settleman, *Factors underlying sensitivity of cancers to small-molecule kinase inhibitors.* Nature Reviews Drug Discovery, 2009. **8**: p. 709.
95. Ledgerwood, L.G., et al., *The degree of intratumor mutational heterogeneity varies by primary tumor sub-site.* Oncotarget, 2016. **7**(19): p. 27185-98.
96. Chong, C.R. and P.A. Jänne, *The quest to overcome resistance to EGFR-targeted therapies in cancer.* Nature Medicine, 2013. **19**: p. 1389.
97. Feng, Z., et al., *Chk1 inhibitor Gö6976 enhances the sensitivity of nasopharyngeal carcinoma cells to radiotherapy and chemotherapy in vitro and in vivo.* Cancer Letters, 2010. **297**(2): p. 190-197.