In vitro Anti-Tumor Efficacy of Trimeric in Head and Neck Cancer

Undergraduate Research Thesis

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by

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Abstract:

In this project, we will determine the anti-tumor activity of trimeric in head and neck squamous cell carcinoma (HNSCC) cells. Trimeric is a novel formulation consisting of three natural products with known anti-cancer activity. The goal of the study was to assess the efficacy of trimeric, as a standard of chemotherapeutic care, to inhibit the viability of HNSCC *in vitro*. Two established HNSCC cell lines, CAL27 and UMSCC-47, and non-tumorigenic human keratinocytes (HaCaT) were treated with trimeric for 24-72 h and dose response curves were generated. The IC50 values for each treatment regimen for HNSCC cells and HaCaT cells were calculated and compared to determine the therapeutic index. It was determined that trimeric showed chemotherapeutic activity in all three cell lines over both 24 and 48 h treatment regimens. In addition, anti-cancer activity was determined in both HNSCC cell lines in the 72 h trial. No significance was found in differential responses between cell lines. We explored the mechanism of action of trimeric with a focus on promotion of cell toxicity. Annexin V-positive apoptotic CAL27 cells substantiated that trimeric induced apoptosis in this HPV-negative cell line.

This proposal has several implications to the field of head and neck squamous cell carcinoma (HNSCC). Our results provide key insight to the efficacy and mechanism of action of trimeric, a novel formulation of three natural products, on HNSCC. These are necessary initial steps for the development of trimeric as a potential anti-cancer therapeutic to manage HNSCC patients.

Introduction:

Head and neck cancer is a broad category of varying tumor types that arise from several anatomic structures including the craniofacial bones, soft tissues, salivary glands, skin, and mucosal membranes. Head and neck squamous cell carcinoma (HNSCC) is a specific subtype that accounts for over 90% of head and neck cancers. To be considered HNSCC, it must occur in the mucosal lining of the upper aerodigestive tract: 1) the nasal cavity and paranasal sinuses, 2) the nasopharynx, 3) the hypopharynx, larynx, and trachea, or 4) the oral cavity and oropharynx. The mucosal membrane consists primarily of the squamous cells for which the disease is named (1).

HNSCC is the sixth leading cancer worldwide and usually develops in males around 60-70 years of age (2). Primary risk factors include smoking tobacco and consuming alcohol. Smoking habits that increase risk are smoking from a young age, smoking for a long duration, deep smoke inhalation, and a large volume of cigarettes per day. Avoiding tobacco and alcohol consumption can prevent up to 90% of HNSCC (3). In addition, there are occupational hazards like working with metal dust, varnish, lacquer, etc., that may lead to a higher risk of developing HNSCC. Recent studies have show that infection with high-risk types of the Human Papilloma Virus (HPV), specifically subtypes HPV16 and HPV18, can cause HNSCC. HPV-positive cases present a unique pathophysiology compared to HPV-negative cases that are worth exploring (4).

SCC can occur in many regions throughout the body with varying degrees of recurrence and risk of metastasis. SCC is the second most common cancer of the skin with the primary risk factor being sunlight exposure. It has low risk of metastasis. However, SCC of the lips and ears are prone to recurrence as well as distant metastasis. Thyroid SCC presents with a very aggressive phenotype with poor prognosis. Prostate SCC also presents with an aggressive

phenotype. This is in part due to the fact that it is difficult to detect and is often diagnosed at an advanced stage, resulting in poor prognosis. Vaginal and cervical SCCs are often associated with HPV. Although they have the tendency to spread slowly, they may metastasize. Treatment generally consists of surgery. SCC of the lung has a five-year survival rate of less than 50%, decreasing steadily the farther along it is. Despite being slow-moving, it still readily metastasizes (4).

The World Health Organization (WHO) classifies eight different types of SCC. Conventional type SCC is seen as keratinization and invasive growth with disruption of the basement membrane. SCCs are categorized by grades of differentiation: well-differentiated, moderately-differentiated, and poorly-differentiated. Well-differentiated SCCs resemble normal squamous mucosal cells. Moderately-differentiated SCCs display nuclear pleomorphism, less keratinization, and mitoses. Poorly-differentiated SCCs primarily consist of immature cells and minimal keratinization. The majority of SCC's are moderately differentiated. Verrucous carcinoma is a well-differentiated, non-metastasizing tumor that can be distinguished by its wart-like, slow-growing nature. Basaloid SCC is a rare, aggressive form that displays hyperchromatic nuclei without nucleoli and little cytoplasm. Due to its rapid growth, it is often diagnosed at an advanced stage with poor prognosis. Papillary SCC presents with an exophytic growth with thin papillary projections. This type generally has a favorable prognosis. Spindle cell carcinoma is a biphasic tumor consisting of both SCC and a malignant spindle cell component. The surface of these tumors is often ulcerated. It also usually has a polypoid appearance with its cells being pleomorphic. Acantholytic SCC is a rare variant that is distinguished by acantholysis, or the loss of intercellular connections, of tumor cells. Adenosquamous carcinoma is another rare aggressive type that contains both SCC and adenocarcinoma. Both components occur next to

each other but tend to remain distinct and separate. The final type is carcinoma cuniculatum, which is a rare variant that can infiltrate bone tissue (5,6).

HNSCC is clinically diagnosed by the severity, location, and histopathology of lesions. Macroscopically, lesions can be described as discrete or diffuse, smooth or irregular, and flat or exophytic. In addition, lesions can be muddy brown to red (erythroplakic), white (leukoplakic), or mixed red and white (speckled leukoplakic). Purely leukoplakic lesions are considered to be low risk to develop into malignancy. Speckled leukoplakic lesions are a moderate risk to undergo a malignant transformation. Pure erythroplakia is the greatest risk to develop into cancer. Despite this seemingly defined method, there is no universally agreed upon classification process to measure dysplasia progression. Likewise, there are many different terms used by clinicians to describe the same thing. For example, dysplasia can also be described as keratosis, squamous intraepithelial neoplasia (SIN), oral intraepithelial neoplasia (OIN), etc. However, efforts to unify language have not been successful, lending to three separate classification systems with different vocabulary and scales. Most clinicians advocate for the combination of two of these systems (8).

According to WHO, there are several histopathological descriptions that grade OINs. These include: 1) loss of polarity of the basal cells, 2) proliferation of the basal cells, 3) increased nucleus-to-cytoplasm ratio, 4) epithelial hyperplasia with drop-shaped submucosal rete extension, 5) irregular epithelial stratification and cellular pleomorphism, 6) premature keratinization of single cells (dyskeratosis) or keratin pearls in the rete pegs, 7) increased mitotic figures and abnormally superficial mitoses, 8) presence of abnormal mitotic figures, 9) variation in nucleus size, shape, and hyperchromatism, 10) increased number and size of nucleoli, and 11) abnormal variation in cell shape and size. Atypical epithelial cell accumulation and SCC is
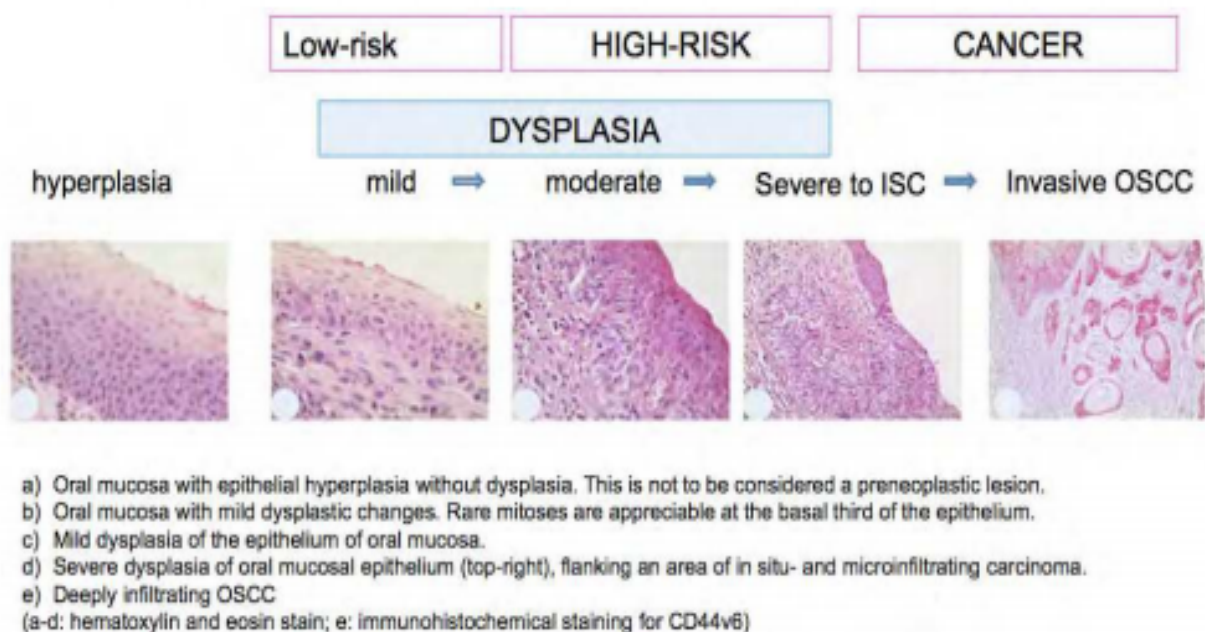
a) Oral mucosa with epithelial hyperplasia without dysplasia. This is not to be considered a preneoplastic lesion.
b) Oral mucosa with mild dysplastic changes. Rare mitoses are appreciable at the basal third of the epithelium.
c) Mild dysplasia of the epithelium of oral mucosa.
d) Severe dysplasia of oral mucosal epithelium (top-right), flanking an area of in situ- and microinfiltrating carcinoma.
e) Deeply infiltrating OSCC
(a-d: hematoxylin and eosin stain; e: immunohistochemical staining for CD44v6)

Figure 1. Progerssion of oral cancer from atypical hyperplasia to invasive carcinoma (8).

related to an increase in frequency of genetic changes that lead to a new population of

transformed normal epithelial cells (8,9). Figure 1 describes grading from atypical hyperplasia to

invasive HNSCC. Atypical hyperplasia describes an increase in cell proliferation. This differs

from dysplasia in that dysplasia describes phenotypic change in cell tissue and is considered a

preneoplastic lesion. Growing thickness in dysplastic cells change diagnosis from mild to either

moderate or severe dysplasia. Full-thickness dysplasia is termed carcinoma *in situ* (CIS). At this

stage, cells have lost tissue identity and undergo rapid growth. However, no invasion of the

basement membrane has occurred. Invasive SCC occurs when proliferation has gone beyond the

basement membrane and there is potential for metastasis. Although this classification was

originally developed for the female genital tract, it has been adapted for all mucosal membranes

(10,11).

The development of HNSCC is a result of both overactivation of proto-oncogenes

stimulating growth and suppression of tumor suppressor genes (TSGs). Califano and colleagues

have shown that the development of HNSCC from benign hyperplasia to CIS and invasive SCC

is the result of progressive genetic alterations. Each histopathological stage of development is

associated with increased chromosomal loss. The earliest and most common alterations occur on

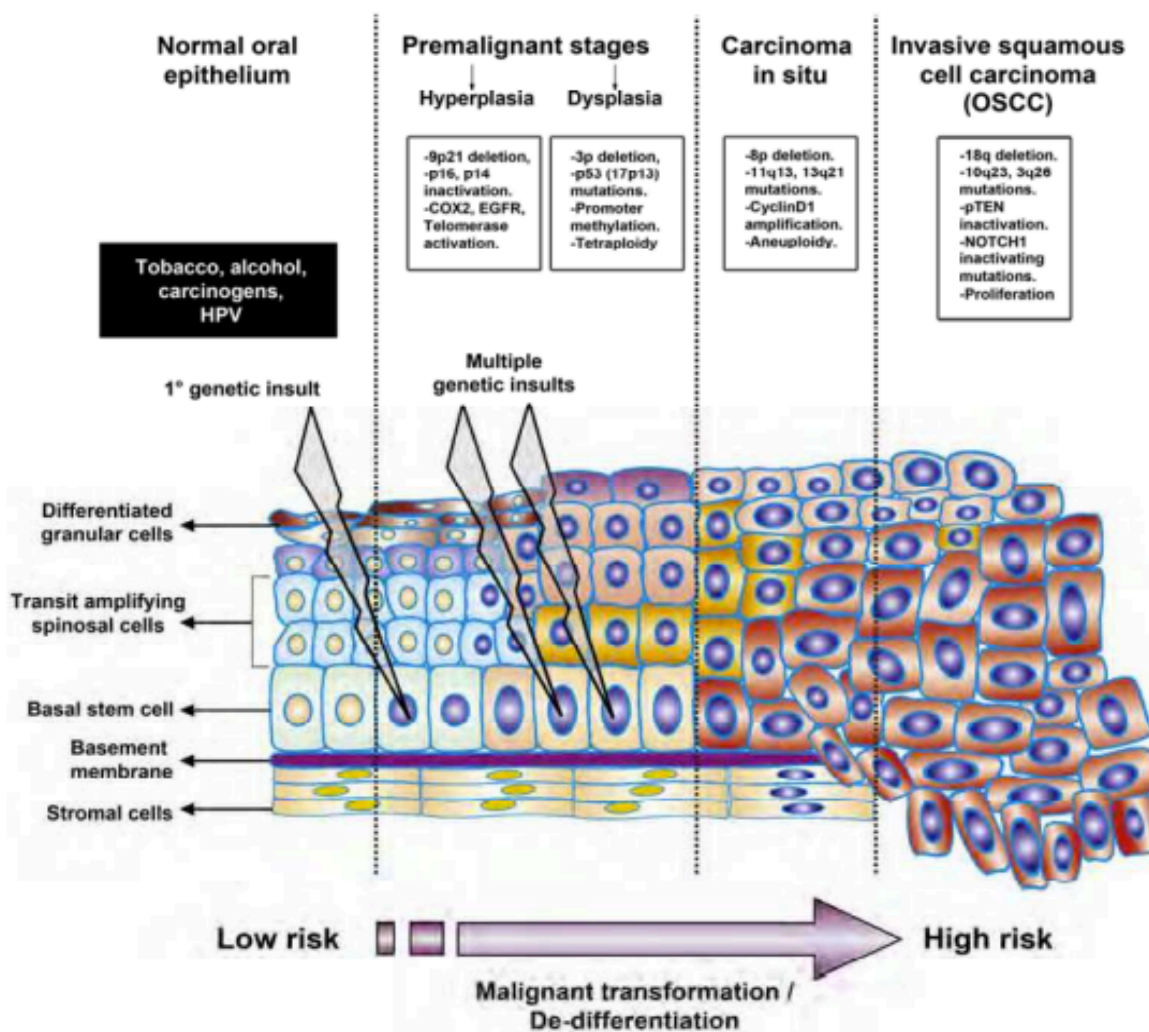chromosomes where p16 and p53 genes are located (12). The p16 gene can be altered in many

Figure 2. Chromosomal deletions and alterations with respect to the development of HNSCC (8).

ways including homozygous deletion, mutation, and promoter hypermethylation. This gene is

integral in cell cycle regulation by slowing down progression of cells from G1 phase to S phase,

thus acting as a TSG (13). Mutation or inactivation of the p53 gene disrupts important cell

activities such as DNA synthesis and repair, gene transcription, and apoptosis, which have been

observed in up to 80 percent of HNSCCs (14). Recent studies have shown that the oncogene Notch-1 is an important factor in the development of HNSCC. Initially acting as a cell surface receptor, ligand binding initiates cleavage of its cytoplasmic tail, which then acts as a transcription factor in the nucleus promoting genes key to cell differentiation, proliferation, and apoptosis (15). In addition to gene mutation, protein overexpression can facilitate cancer progression. Epidermal growth factor receptor (EGFR) is present at elevated levels in over 90 percent of HNSCCs and is integral to progressing intraepithelial lesions to SCC. EGFR is a growth-regulating receptor glycoprotein that is influential in cell division, migration, adhesion, differentiation and apoptosis through a tyrosine kinase pathway (15,16). Although there are many other chromosomal mutations and changes in protein expression that could proliferate HNSCC, these occur most often and are the primary targets worth investigating.

As mentioned previously, HPV is identified as a major risk factor for developing oropharyngeal SCC (OPSCC), a specific subtype of HNSCC. With incidences of HPV infection increasing at an alarming rate (approx. three-fold) over the last thirty years, there is a clinical need to develop therapeutic drugs to deal with the growing numbers of HPV-positive HNSCC. Specifically HPV16 is the most prevalent subtype, present in approximately 90 percent of all HPV-positive HNSCC (17,18). Both HPV-positive and HPV-negative cells have unique histological profiles that require separate and distinct treatment strategies. HPV produces two oncoproteins that effect cell proliferation: E6 inactivates p53 via proteasomal degradation, and E7 competes for binding with the retinoblastoma protein (pRb), which is responsible for inhibiting cell cycle progression (19). One example of histological differences dependent on HPV infection is that the p53 gene is primarily wild-type in HPV-positive HNSCC, while it is predominately mutated in HPV-negative HNSCC (21). Another example is with regard to cancer

stem cells (CSCs). CSCs are resistant to conventional therapies and are thought to potentially be responsible for disease recurrence (20). A study by Zhang et al. demonstrates that intrinsic CSC levels are higher in HPV16-positive than HPV-negative OPSCC tumors. Since HPV16-positive OPSCC has a better clinical outcome, it was hypothesized that this was possibly due to HPV16-positive OPSCC having a lower number of CSCs than HPV-negative OPSCC. However this was not the case as HPV16-positive OPSCC has higher intrinsic CSCs than HPV16-negative OPSCC. This implies that the phenotypes of HPV-positive and HPV-negative CSCs are not homogenous and may be more important than the actual number of CSCs as a marker for an aggressive disease (28).

High risk HPV E6 inactivates p53 via two mechanisms: 1) association with E6AP to degrade p53 and 2) association with p300 to block p300-mediated p53 acetylation. Acetylation of p53 increases its stability and transcriptional activity (21-23). Xie et al. took a novel approach by focusing on the association of high risk E6 with p300. By ectopically expressing the CH1 domain on p300, E6-p300 interaction was disrupted resulting in elevated p53 acetylation, accumulation, and activity. Furthermore, treatment with a CH1 inhibitor increased the effectiveness of cisplatin, a frequently used chemotherapeutic, showing the efficacy of combination therapy (24).

An additional example of the effectiveness of combination therapy is inhibition of EGFR. Only 5-15% of patients respond to anti-EGFR treatments indicating that inhibition of EGFR tyrosine kinase-dependent activity and downstream signaling may not be the most effective treatment (25). Although EGFR is responsible for many downstream signaling pathways, related surface receptors such as HER2 and HER3 can also signal to the same downstream proteins (26). This can explain why such a low percentage of patients respond to single-agent anti-EGFR

treatment. Epithelial-restricted with serine box (ESX) is a transcription factor that was known to

have a positive feedback loop with HER2 and has been linked to controlling apoptosis, cell

differentiation, and proliferation. Zhang and colleagues were able to show that it also had a link

to EGFR promoter activity. Genetic ablation of ESX reduced EGFR and HER2 levels and

inhibited cell proliferation, invasion, migration, and clonogenic survival. Thus, using a mimic of

ESX showed anti-tumor response as monotherapy, but was even more effective in combination
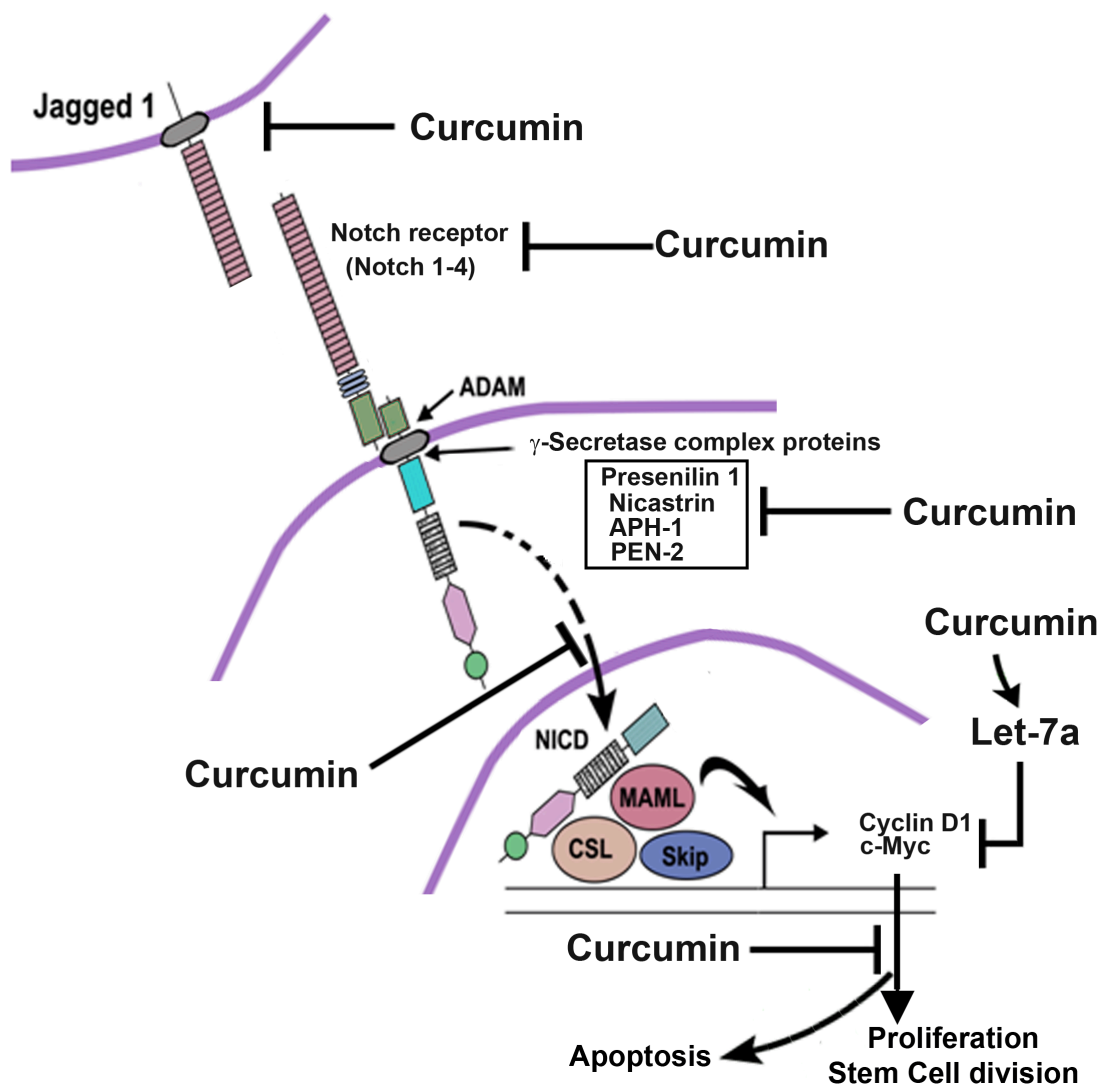
with afatinib, a tyrosine kinase inhibitor (16).

Figure 3. Subramaniam et al. determined that curcumin inhibited both Notch-1 and Jagged 1 expression, as well as γ-secretase proteins, preventing cleavage of Notch-1. As a result, Notch intracellular domain (NICD) is not translocated into the nucleus to activate proliferation-stimulating genes. Thus, proliferation and stem cell division are reduced while apoptosis is induced (30).

Current treatment methods are evolving from traditional monotherapy to combination therapy involving multiple target proteins or genes. Trimeric is a type of combination therapy that is a novel formulation of three drugs with known chemotherapeutic activity. One known ingredient in this mixture is curcumin, the active ingredient in curry powder. Curcumin, a pigment derived from turmeric, has been shown to have multiple anticancer effects, including inhibition of proliferation, induction of apoptosis, inhibition of angiogenesis, and inhibition of DNA topoisomerase II. Curcumin also has been shown to induce apoptosis-independent death such as autophagy in esophageal cancer cells (29). Subramaniam et al. determined that curcumin downregulated Notch-1 cell-surface receptor as well as its ligand, Jagged 1. It was also responsible for inhibiting Notch-1 downstream signaling and target genes, further inhibiting proliferation and inducing apoptosis. Thus, it was shown that curcumin has anti-cancer capabilities and is a strong candidate for therapeutic treatment of esophageal cancer (30).

Liao, Xia, et al. showed the inhibitory effect of curcumin on CAL27 cells via inhibition of Notch signaling and NF-ϰB, which is regulated by Notch-1. It was also determined to induce apoptosis (31). Thus as curcumin has been shown to induce apoptosis in CAL27 cells, it is important to determine the effectiveness of curcumin as an ingredient in trimeric. Furthermore, this study will explore the efficacy of trimeric with regards to HaCaT and HPV-positive and HPV-negative HNSCC cell lines, the relative dose-responses, and mechanism of action.

Materials and Methods:

Cell Lines

CAL27 cells were purchased from the American Type Culture Collection (Manassas, Va).

UMSCC-47 cells were obtained from Dr. Thomas Carey at the University of Michigan.

HACAT*** CAL27 and UMSCC-47 cells were grown in DMEM containing 10% FBS, 2 mM

L-glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin. **HACAT******

Cell proliferation

To assess proliferation, cells were seeded with a density of 3000 cells/well on 96-well plates and

grown overnight. Then the cells were treated with 0.025, 1.0, 4.0, 16.0, and 64.0 μM trimeric for

24, 48, and 72 h. Each concentration of trimeric was diluted in appropriate media and the control

group was treated with RPMI. After treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) solution (5 mg/ml in phosphate-buffered saline, PBS) were

added to each well and incubated for 2 h. **READINGS WERE DONE ON*****

Apoptosis

For apoptosis, cells (~1x10^6) were treated with trimeric for 24 hours at their respective IC50

values, collected, washed with cold phosphate-buffered saline, and costained with annexin V and

propidium iodide according to the manufacturer's protocol (ApoAlert Annexin V-FITC

Apoptosis Kit; Clontech, Mountain View, CA, USA). Apoptotic cells were analyzed using BD

FACS Calibur (BD Biosciences Corporation, Franklin Lakes, NJ, USA) at the Ohio State

University Comprehensive Cancer Center Analytical Cytometry Core.

Results:

Treatment with trimeric inhibited proliferation in all cell lines. In Figure 4 below, IC50 values for each cell line are displayed below their respective dose-response curves. There seemed to be no significance for different IC50 values between the cell lines and across all treatment periods. To measure apoptosis, cells were treated with trimeric at their respective 24 h-IC50 values for 24 h (Figure 5). By staining both control and treated cells with annexin V-FITC and PI, we found that the percentage of annexin V-positive apoptotic cells. CAL27 is the only cell line that indicates that apoptosis was triggered by trimeric, increasing from 10.62% to 76.8% apoptotic cells. It is unclear as to the mechanism of cell death in both UMSCC-47 and HaCaT, however, staining does show a right-shift in the PI-negative, FITC-negative control group populations for both of these cell lines. Due to the high percentage of annexin V-positive, apoptotic cell lines in the control groups of HaCaT and UMSCC-47, it is difficult to determine the significance of the right-shift.
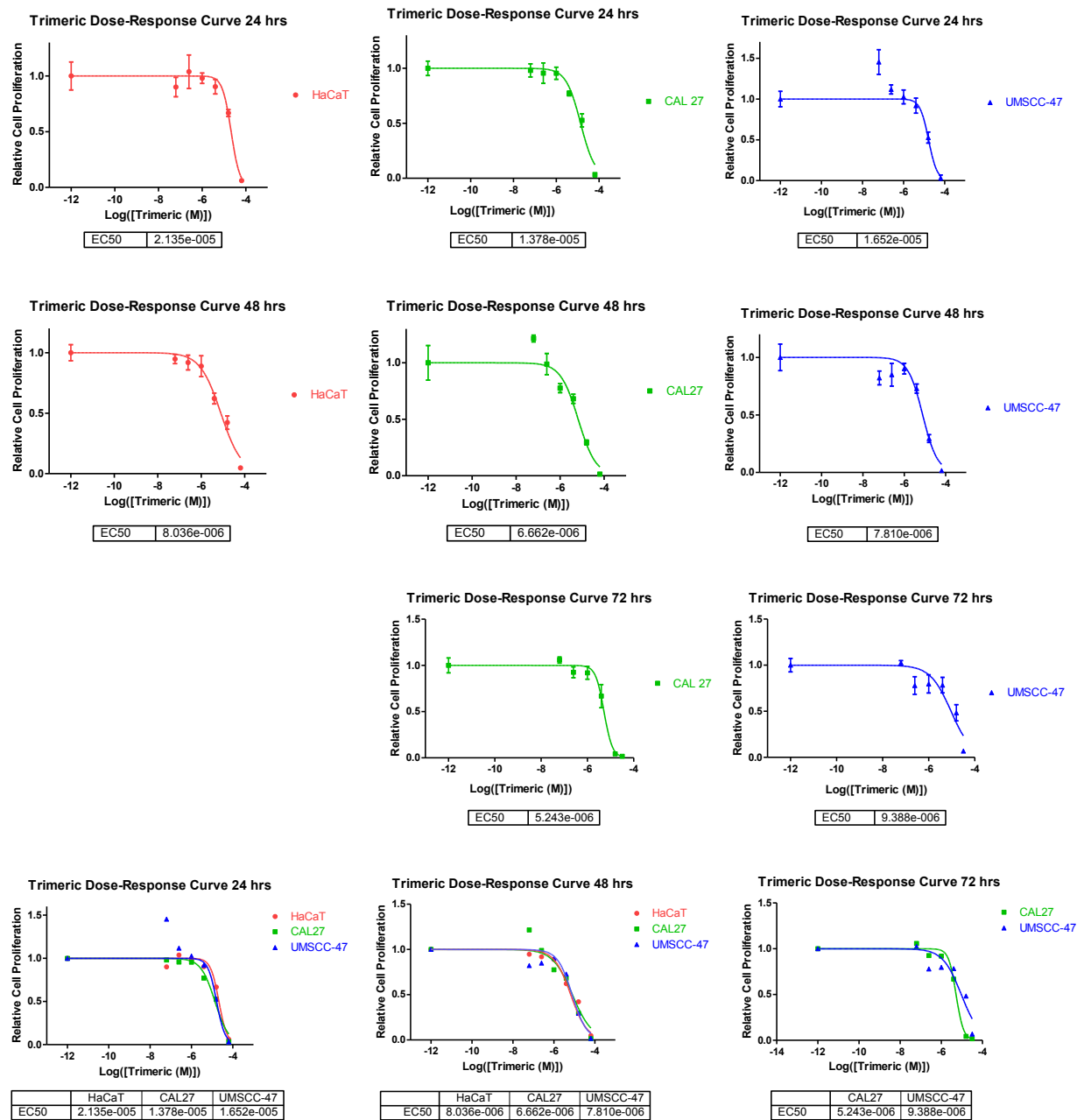
Figure 4. Each graph is specific for time and cell line type. The first three rows depict dose responses at all three time intervals (Row 1: 24 h, Row 2: 48 h, Row 3: 72 h). Each column designates each cell line (Left: HaCaT, Middle: CAL27, Right: UMSCC-47). There was insufficient data for HaCaT treatment for 72 h. The bottom row represents the synthesis of all three cell lines for the respective time intervals. IC50 (EC50) values are displayed below each graph.
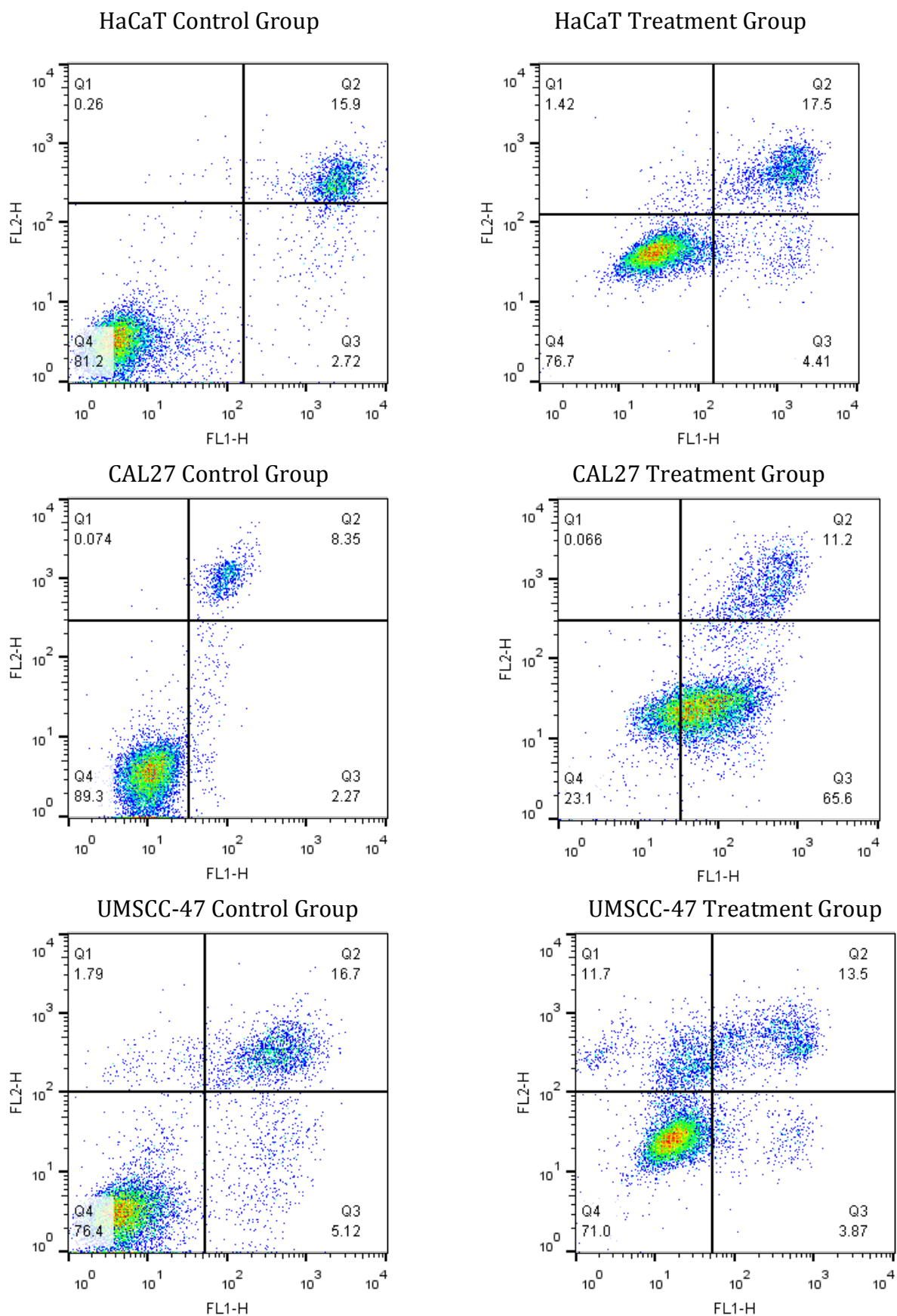
Figure 5. Trimeric induced apoptotic death in CAL27. It is unclear as to the method of cell death for both UMSCC-47 and HaCaT.

Discussion:

  Trimeric showed chemotherapeutic activity in all cell lines across all treatment intervals indicating a potential for anti-cancer activity. However, cell lines showed no differential sensitivity to trimeric, showing evidence that it may not be viable for clinical use.

  HaCaT cells showed an upward- and right-shift in staining after treatment with trimeric. Data indicates an insignificant shift due to the high population of annexin V-positive cells in the control group. However, there is a tenfold increase in both PI and FITC staining after treatment, indicating that trimeric may be inducing cell death via some method. Additionally, UMSCC-47 cells experienced the same problem: there was a high percentage of annexin V-positive cells in the control group. This time there was a tenfold increase in PI staining after treatment, indicating that trimeric could be inducting cell death via membrane disruption, possibly necrosis. CAL27 showed a significant shift in both PI and FITC staining, clearly indicating that trimeric induces apoptosis in CAL27 cells.

  Comparing results of CAL27 treated with trimeric with the results of Liao, Xia et al. show very similar results for IC50 values and percentage of annexin V-positive apoptotic cells. This makes it unclear as to whether or not the other active ingredients in trimeric potentiates the efficacy of curcumin. Curcumin has the unique property of protecting non-transformed cells and tissues from the damaging effects of ionizing radiation, while acting as a radiation sensitizer of malignant cancer cells (32). Tuttle et al. determined that curcumin potentiated the efficacy of irradiation in HPV-negative cells lines; however the sensitivity to irradiation was not improved in combination with curcumin for HPV-negative cell lines (33). We hypothesize that proliferation of CAL27 could be better inhibited with combination therapy of irradiation and trimeric treatment. Based on this evidence, UMSCC-47 may not be affected by combination

therapy as a result of curcumin; however, it could be substantiated by the other active ingredients.

Future Research:

Further research will explore the efficacy of trimeric in xenograft mice models. Additionally, combination therapies with known chemotherapeutics like cisplatin and affatinib should be carried out. Further in-depth investigations together with pre-clinical animal studies are needed to establish how curcumin induces cell growth inhibition and apoptosis in HNSCC. Additionally, as this study only used one HPV-positive (UMSCC-47), one HPV-negative (CAL27), and one non-tumorigenic human keratinocyte (HaCaT) cell lines, a wider range of tumor models are need to draw better conclusions on differential efficacy based on HPV status. For example, an HPV-positive, p53-mutated cell line as well as HPV-negative, p53 wild-type cell line should be examined.

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