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Benzyl Isothiocyanate as an Adjuvant Chemotherapy Option for Head and Neck Squamous Cell Carcinoma

Mary Allison Wolf
teter6@marshall.edu

Benzyl Isothiocyanate as an Adjuvant Chemotherapy Option for Head and Neck Squamous Cell Carcinoma

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By
Mary Allison Wolf

Approved by
Pier Paolo Claudio, M.D., Ph.D., Committee Chairperson
Richard Egleton, Ph.D.
W. Elaine Hardman, Ph.D.
Jagan Valluri, Ph.D.
Hongwei Yu, PhD

Marshall University
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DEDICATION

"I sustain myself with the love of family"—Maya Angelou

To my wonderful husband, loving parents, and beautiful daughter—thank you for everything you have given me.

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LIST OF ABREVIATIONS

4E-BP	—	4E-Binding protein
AIF	—	Apoptosis inducing factor
ALDH-1	—	Aldehyde dehydrogenase-1
AUC	—	Area under the curve
Bak	—	Bcl-2- antagonist/killer-1
Bax	—	Bcl-2-associated X protein
Bcl-2	—	B-cell lymphoma-2
Bcl-xL	—	B-cell lymphoma-2-extra large
BITC	—	Benzyl isothiocyanate
Cdc2	—	Cell division cycle 2
Cdc25B	—	Cell division cycle 25B
CDDP	—	cis-diamminedichloroplatinum(II)
Cdk1	—	Cyclin-dependent kinase 1Cell division cycle 2
Cis	—	Cisplatin
CSC	—	Cancer stem cell
CYP450	—	Cytochrome p450
DMEM	—	Dulbecco's Modified Eagle Medium
EGF	—	Epidermal growth factor
EGFR	—	Epidermal growth factor receptor
EMT	—	Epithelial-mesenchymal transition
endoG	—	Endonuclease G
ERK	—	Extracellular signal-regulated kinases
FBS	—	Fetal bovine serum
G-CSF	—	Granulocyte colony-stimulating factor
GSH	—	Glutathione
GST	—	Glutathione S Transferase
H_2O_2	—	Hydrogen Peroxide
HDAC	—	Histone deacetylase
HIF-1α	—	Hypoxia inducible factor-1α
HNSCC	—	Head and neck squamous cell carcinoma
HPLC	—	High-performance liquid chromatography
i.v.	—	Intravenous
IL-6	—	Interleukin-6
ITC	—	Isothiocyanate
JNK	—	c-Jun N-terminal kinase
Keap-1	—	Kelch-like ECH protein 1
LC-MS/MS	—	Liquid chromatography/ tandem mass spectrometry
MAPK	—	Mitogen-Activated Protein Kinases

NAC	—	*N*-acetyl-L cysteine
NFκB	—	Nuclear factor kappa-light-chain-enhancer of activated B cells
NNK	—	Nicotine-derived nitrosamine ketone
Nrf2	—	Nuclear factor erythroid 2 related factor 2
NSCLC	—	Non-small cell lung cancer
p.o.	—	Per os
p16	—	Cyclin-dependent kinase inhibitor 2A
p21/WAF1	—	Cyclin-dependent kinase inhibitor 1
p38	—	p38 mitogen-activated protein kinases
p53	—	Tumor protein p53
PARP	—	Poly ADP ribose polymerase
PBS	—	Phosphate Buffered Saline
PEITC	—	Phenethyl isothiocyanate
pRB	—	Retinoblastoma protein
ROS	—	Reactive oxygen species
SFN	—	Sulforaphane
STAT-3	—	Signal transducer and activator of transcription 3
TRAIL	—	TNF-related apoptosis-inducing ligand
VEGF	—	Vascular endothelial growth factor

ABSTRACT

Isothiocyanates (ITCs) are natural phytochemicals produced by cruciferous vegetables. Recent evidence supports that, in addition to cancer prevention, ITCs can use various mechanisms to target malignant cells. Current therapies for cancer often provoke detrimental side effects, however clinical evidence supports that ITCs have little to no side effects in patients. Consequently, ITCs may be a promising treatment option for cancer patients, especially patients suffering from head and neck squamous cell carcinoma (HNSCC).

Despite recent improvements in cancer treatment, overall survival of advanced HNSCC has not improved in the past three decades. Metastasis and chemoresistance represent two detrimental events that greatly hinder the outcome for those suffering with HNSCC. Thus, new therapeutic options to enhance survival of patients with advanced HNSCC are needed. Several types of ITCs can be used to target HNSCC, however our studies indicated that benzyl isothiocyanate (BITC) elicits the strongest anti-tumor response when targeting chemoresistant and metastatic HNSCC cell lines.

In our *in vitro* studies, we evaluated the use of BITC as a treatment for HNSCC. Our study had three objectives; the first being to investigate if this compound can prevent HNSCC cell migration and invasion, the second was to study if BITC could enhance the effects of chemotherapy, and the third was to identify a mechanism through which BITC was eliciting its anti-tumor response.

Our *in vitro* data suggests that treatment with BITC significantly reduced the viability of multiple HNSCC cell lines tested (HN12, HN8, and HN30), but not a normal keratinocyte cell line (HAK). BITC treatments also decreased the migration and invasion of the HN12 cell line, in a dose dependent manner, at concentrations that did not affect cell viability. Additionally, when

compared to either BITC or cisplatin treatment alone, the reduction in HNSCC cell viability was greater if a pretreatment of BITC was followed by a treatment of cisplatin.

Furthermore, the expression of the epithelial-mesenchymal transition (EMT) marker, vimentin, was significantly reduced after a BITC treatment in the HN12 cell line. We also observed that BITC treatments significantly increased the amount of reactive oxygen species (ROS) in HNSCC cells. Blocking BITC induced ROS with co-administration of catalase or *N*-acetyl-L-cysteine (NAC) significantly inhibited BITC's ability to prevent cellular migration. Co-administration of NAC with BITC prior to cisplatin treatment reduced cytotoxicity as compared to BITC pre-treatment followed by cisplatin. Therefore, indicating that co-administration of anti-oxidants with BITC could alter the clinical efficacy BITC.

Taken together these data suggest that BITC has the capacity to inhibit processes involved in HNSCC cell migration and invasion, as well as add to the effectiveness of chemotherapy, and both of these events are regulated by BITC induced ROS.

Chapter 1 **: Isothiocyanates Target Carcinogenesis during Tumor Initiation, Promotion, and Progression**

This manuscript is a revised version of Chapter 9 from *Nutrition and Cancer, From Epidemiology to Biology* (2013).

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M. Allison Wolf [1] and Pier Paolo Claudio [1,2,3]

Marshall University School of Medicine, Huntington, WV
[1]Department of Biochemistry and Microbiology
[2]Department of Surgery

[3]Corresponding author
Marshall University School of Medicine – Translational Genomic Research Institute
Department of Biochemistry and Microbiology
1600 Medical Center Drive, Huntington, WV, 25701
Phone: 304-696-3516
FAX: 304-696-3713
Email: claudiop@marshall.edu

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ABSTRACT

Isothiocyanates (ITCs) are phytochemicals produced from the hydrolysis of glucosinolates, which are found at high concentrations in cruciferous vegetables. Vegetables of the Cruciferae family include broccoli, cauliflower, gardencress, watercress, and cabbage. A number of studies have suggested that the ITCs, sulforaphane (SFN), benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC) are capable of preventing breast, lung, and prostate carcinogenesis. In addition to chemoprevention, SFN, PEITC, and BITC have also been investigated for their chemotherapeutic potential in numerous cancers. The anti-cancer effects of SFN, BITC, and PEITC are associated with several cellular pathways that inhibit growth, induce apoptosis, and prevent metastasis.

Studies investigating the chemopreventive and chemotherapeutic properties of SFN, PEITC, and BITC are quickly progressing from bench to beside. This chapter will give a brief overview of SFN, PEITC, and BITC, focusing mainly on these ITC's reported ability to inhibit carcinogenesis *in vivo* and *in vitro* during three stages: initiation, promotion, and progression.

INTRODUCTION

Isothiocyanates (ITCs) are phytochemicals produced by vegetables in the Cruciferae family [1-3]. Cruciferous vegetables include broccoli, Indian cress, cabbage, Brussels sprouts, and watercress [4]. ITCs are generated naturally from the hydrolysis of glucosinolates, which are a secondary metabolite found in cruciferous vegetables, and contain a β-D-thioglucose group, a sulfonated oxime moiety, and a variable side chain [4]. A cruciferous vegetable produces ITCs as a defense mechanism when the plant is damaged or "under attack". This family of vegetables spatially separates glucosinolates (cytoplasm) from the defense-related enzyme myrosinase (external surface of the plant cell wall), and when the plant is damaged or chewed the enzyme and glucosinolate are brought into contact, and the glucosinolate undergoes a Lossen rearrangement creating the ITC product [5]. The glucosinolate precursor dictates the type of ITC produced (**Table 1.1**). There are currently over a 100 glucosinolates identified, but not all of the corresponding ITCs appear to have anti-carcinogenic properties [2]. Examination of the literature suggests that the ITCs most frequently investigated for their anti-cancer effects are sulforaphane (SFN), benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC), and are therefore the main focus of this chapter.

Table 1.1 Isothiocyanates with their corresponding glucosinolate precursor and food source.

Isothiocyanate	Glucosinolate	Food Sources
Benzyl Isothiocyanate (BITC)	Glucotropaeolin	Cabbage, garden cress, Indian cress
Phenethyl Isothiocyanate (PEITC)	Gluconasturtiin	Watercress
Sulforaphane (SFN)	Glucoraphanin	Broccoli, Brussels sprouts, cabbage

All ITCs, including SFN, BITC, and PEITC have the same R-N=C=S structure (**Figure 1.1**). The reactive group is the sulfur containing N=C=S functional group, which is a strong

electrophile and can undergo a nucleophilic attack [6]. The N=C=S group is reported to selectively bind to thiol-containing cysteines and ε-amino containing lysines forming thiocarbamates and thioureas, respectively [7]. ITCs are suggested to form thiocarbamates at a 10^3 to 10^4 faster rate than thioureas, but are less stable [6,7]. The ability of ITCs to target cysteine residues is significant, because cysteine residues are often found in the catalytic site of enzymes [5]. The binding to cysteine residues is suggested to be one way in which ITCs can alter signal transduction and redox status [2].

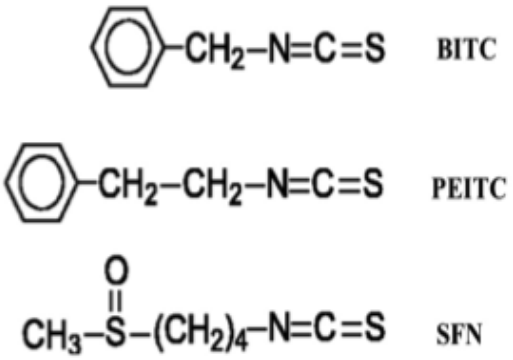

Figure 1.1 Chemical structure of BITC, PEITC, SFN.

The R group of ITCs varies significantly, and can be either an alkyl or aryl group. For example, SFN contains an alkyl side chain, whereas PEITC and BITC's side chain is an aryl group (**Figure 1.1**). Proteomics studies have indicated that the R group may play an important role in the targets of ITCs [7,8]. In A549 cells ^{14}C -SFN was shown to bind to only 16 proteins, whereas ^{14}C-PEITC was shown to target more than 30 proteins [7]. Additionally, a difference in the mechanism of action has been reported when investigating PEITC and SFN's involvement in cell cycle and apoptosis [5,6]. Furthermore, PEITC and BITC are reported to inhibit cellular

proliferation, induce apoptosis, and inhibit cellular migration at significantly lower concentrations than SFN [3,9-11]. This has also been supported in animal models [2,4,12].

Regardless of the mechanism of action, SFN, BITC, and PEITC have all been shown to inhibit the growth of many cancerous cell lines, including lung, prostate, and breast [13-17]. However, when considering an effective treatment for any disease one must assess whether or not the treatment is feasible in humans. Many phytochemicals or polyphenols have indicated promising results in cell culture, but the serum concentrations needed to observe similar effects in humans has not been achieved. Studies indicate that the oral bioavailabilities of ITCs are high; suggesting that they may be a better treatment option than other phytochemicals.

The bioavailability of SFN is reported to be high in animals and humans; however there is limited data on the oral bioavailability in humans. In male Wistar rats 82% of SFN has been shown to be bioavailable [4,18]. PEITC is also suggested to have a high oral bioavailability in both animals and humans [5]. Additionally, the AUC per os (p.o.) and intravenous (i.v.) administration of PEITC does not appear to differ significantly in mice and rats [5,18]. Bioavailability of BITC has only been reported in animals. More than 98% of [7-^{14}C] BITC was rapidly absorbed following oral administration to Wistar rats [19].

There are significant differences when comparing the half-life and AUC between the different ITCs. The half-life and AUC of SFN is lower than that reported of PEITC. In humans, PEITC has a half-life of 3.7 to 4.9 hours; whereas the half-life of SFN is only reported to be 1.8 hrs and 1-2 hours, respectively [5,19]. The half-life of BITC in rats is 1-2 hours [19] . Interestingly, increasing the dose of PEITC increases the half-life, and decreases the clearance [2,4,5]. ITC metabolites are secreted in the urine and the saturation of the enzymes involved in the metabolism of ITCs may be an explanation for the decreased clearance [4].

Additionally, the accumulation of all ITCs into cells is suggested to be rapid, and the intracellular concentration is reported to be several hundred-fold greater than the extracellular concentration [2]. This observation has been supported in mouse pancreatic endothelial and fibroblast cells, as well as in human prostate and colon cancer cell lines [2,5]. ITCs are also reported to reach many tissues because they are coupled to serum albumin and can be systemically transported throughout the body and released into tissues [5].

When considering the transition from bench to bedside, bioavailability is critical for the success of ITCs, but methods to determine ITC concentrations in humans are still being fine-tuned. The problem with many of the original methods used for ITC quantification was that they could only determine total ITC concentration and not specific ITC conjugates. Also, the method for determining ITC concentration in the urine *vs*. the blood may need to be different [2,5]. ITCs are excreted mainly in the urine; therefore the ITC concentration in the urine is very high. The concentration in the blood is much lower, and the detection method in the serum needs to be more sensitive to be able to detect the low levels of ITCs [20]. The test also needs to be specific for a particular ITC, not just ITCs in general. Cyclocondensation is a sensitive test used to detect ITCs; however, it can only identify the total ITC concentration [5]. This reaction also cannot distinguish between ITCs and other thionyl compounds. When using blood samples, the cyclocondensation reaction is not sensitive enough for ITC quantification. An assay using polyethylene glycol followed by membrane ultrafiltration to remove proteins before high-performance liquid chromatography (HPLC) is suggested to be better for ITC identification in blood [5]. Another method for ITC quantification, which can be used for both urine and plasma, involves an ammonia derivation of ITC to phenethylthiourea and subsequent liquid chromatography/tandem mass spectrometry (LC-MS/MS) [5]. The advantage of this method is that it allows specific ITCs to be

identified. This section highlights that, while research on SFN, PEITC, and BITC has come a long way, a better understanding of the pharmacokinetics and pharmacodynamics, as well methods of detection are still needed to advance the clinical aspects of ITC research.

Cancer is a multi-stage process involving initiation, promotion and progression and ITCs are suggested to target each of these stages. The objective for the remainder of this chapter is to briefly review the evidence for the benefits SFN, BITC, and PEITC treatment in both preventing and treating multiple cancer types, as well as a rational mechanism for these effects.

Mechanisms of Action

The mechanism of action involved in the anti-carcinogenic and anti-tumor activity of ITCs have not been fully elucidated. However, the mechanism involved in ITCs ability to inhibit carcinogenesis is more fully understood and involves the inhibition of carcinogen activation. ITCs are known to inhibit the activity of several cytochrome p450s (CYP450), which are Phase I enzymes involved in normal metabolism, and also in carcinogen activation [1,6,21]. By inhibiting the activity of CYP450s, ITCs can prevent DNA adduct formation and the subsequent mutation leading to a transformed cell, thereby preventing carcinogenesis. ITCs also induce certain Phase II enzymes, like GST, through activation of the Keap-1/Nrf2 pathway [2,6]. The induction of Phase II enzymes helps dispose of activated carcinogens by transforming the carcinogen into a water-soluble compound that can be excreted via the urine [6]. Additionally, ITC treatments are suggested to rapidly decrease the concentration of reduced glutathione (GSH) in cells, which can prevent tumor formation [22]. Hyperplasic cells usually have mutations that lead to an increase of reactive oxygen species (ROS) and are sensitive to

GSH depletion [23]. A depletion of GSH can induce apoptosis, thereby preventing the progression of a pre-neoplastic lesion to a malignant tumor.

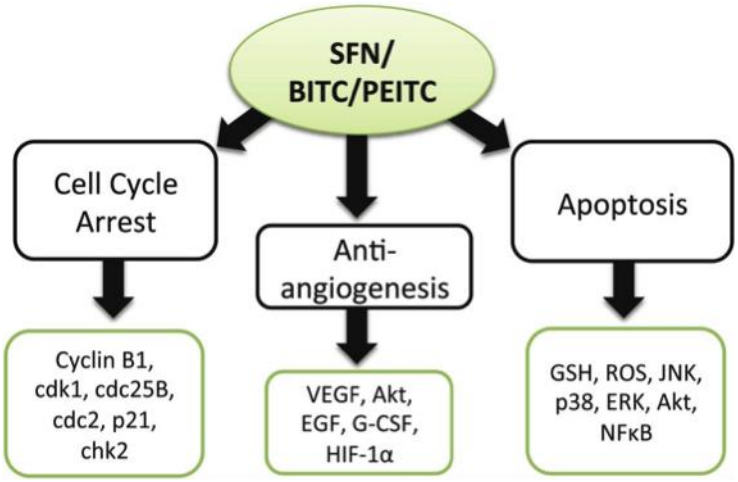

Figure 1.2 Molecular pathways targeted by SFN, BITC, and PEITC.
The anti-tumor activity of these ITCs affect various molecular targets that inhibit cell cycle and angiogenesis, and induce apoptosis.

The mechanisms involved in the anti-tumor activity of ITCs are more complex than the mechanisms linked to chemoprevention. In cancerous cells, ITCs target several different molecular pathways linked to apoptosis, cell cycle arrest, and migration [2,4,24,25]. However, evidence supports that the type of ITC, as well as the concentration, has a significant effect on the mechanism of action. The protein kinase B (AKT), extracellular signal-regulated kinases (ERK), and p38 MAP kinase (p38) pathways are frequently discussed in relation to SFN, PEITC, and BITC treatment [1,2,4]. Alterations in additional pathways are also linked to ITC therapy, as highlighted in **Figure 1.2**, and will be discussed more thoroughly in the following sections [1,2].

The mechanism involved in ITC's ability to trigger apoptosis is complex and therefore deserves some discussion. SFN, PEITC, and BITC induce apoptosis in numerous cancer cell lines; however, induction of apoptosis is different between the treatments [4]. PEITC and BITC can initiate apoptosis by binding to tubulin [4,7]. Dysfunction of tubulin increases the cleavage of caspase-8 and-9. SFN does not have a strong affinity for tubulin, but does induce ROS more potently that PEITC. SFN, PEITC, and BITC are all shown to decrease the anti-apoptotic proteins Bcl-2 and Bcl-xL [4,26]. Additionally, these ITCs induce caspase-3 activity and PARP cleavage [1,27,28]. Studies on PEITC suggest that this ITC has the ability to stimulate caspase-3, -8, and-9 [2]. However, in leukemia cells, it has been shown that caspase-8 is critical and caspase-3 only provides a supporting role [29]. Therefore, studies support that treatment with ITCs initiate apoptosis through both extrinsic and intrinsic mechanisms. The pro-apoptotic protein BID also appears to be cleaved in response to ITC treatment indicating that the c-Jun terminal kinase (JNK) pathway is a target of ITCs [4,30]. SFN, BITC, and PEITC, have all been shown to target the JNK pathway in various cancer cells. Interestingly, in OVAR-3 cells, PEITC suppressed the activation of Akt and ERK1/2, but activated the p38 and JNK1/2 pathway.

Treatments with SFN, PEITC, or BITC induce cell cycle arrest, but literature varies on the type of cell cycle arrest initiated by each ITC. SFN and BITC are both reported to induce G2/M arrest, while PEITC is reported to induce G0/G1 arrest [25,27,31]. However, these studies were done on different cancer cell lines with different concentrations that could account for the different results. In all reported cases, cell cycle arrest after ITC treatment is associated with an increase in cyclin-dependent kinase inhibitor 1 (p21/WAF1) and checkpoint kinase 2 (chk2) [25,27,31,32]. SFN, PEITC and BITC are also all reported to down-regulate expression of

cdc25c, cyclin D1, and cyclin B1 [25,27,31,32]. Additionally, PEITC is reported to down-regulate cyclin A, and SFN and PEITC are both reported to inhibit cyclin E [3,27,31,32].

The mechanisms involved in the inhibition of angiogenesis after ITC treatment are associated with some of the same pathways linked to apoptosis induction. ITCs are known to inhibit the Akt pathway, and this pathway activates mTOR, which consequently activates the 4E-Binding protein (4E-BP). The 4E-BP regulates expression of HIF-1α. SFN, PEITC, and BITC inhibit HIF-1α expression and subsequently the factors regulated by HIF-1α, which are involved in angiogenesis and epithelial-mesenchymal transition (EMT) [17,33,34]. Treatment of prostate cancer PC-3 cells with PEITC also decreased expression of the angiogenic factors, epidermal growth factor and colony-stimulating factor [24]. PEITC and BITC treatment both cause a decrease in the expression of vascular endothelial growth factor (VEGF) [35].

SFN, PEITC, and BITC have all been shown to inhibit migration and invasion of cancer cells *in vitro* [13,33,34,36,37]. SFN, PEITC, and BITC have also been shown to suppress the metastatic potential of breast and NSCLC cells *in vivo* [38-42]. Suppression of metastasis is suggested to occur through modulation of metastasis-related gene expression and inhibition of the Akt/ NF-κB pathway. All three ITCs have been demonstrated to inhibit NF-κB activity in a dose dependent manner. The inhibition of NF-κB activity appears to be a critical target of ITCs, in some cancer cell lines. Inhibition of NF-κB can lead to the inhibition of cell growth, induce apoptosis, and inhibit migration. However, our results suggest that BITC treatment increases NF-κBp50 activity in HNSCC cell lines, indicating that the link between NF-κB activity and ITC treatment is not clear cut.

Uses in Cancer Therapy

Traditional anti-cancer treatments are associated with debilitating side effects. Using ITCs as a chemotherapy or adjuvant therapy is a novel way to target cancer without the associated side effects linked to most cancer treatment. ITC treatments alone are reported to inhibit cell growth, induce apoptosis, and decrease metastasis *in vivo* and *in vitro* [2]. As an adjuvant therapy ITCs sensitize cancer cells to chemo and radiation therapy [28,29,43-47]. ITCs also target cancer stem cell (CSC) populations, which are the major cause for cancer recurrence and drug resistance [2,43,48]. According to the ‘‘cancer stem cell” theory, tumors are not to be viewed as simple monoclonal expansions of transformed cells, but rather as complex tissues where abnormal growth originates from a pathological minority of cancer stem cells [48]. These cells have maintained stem-like characteristics in that they proliferate very slowly and have an inherent capacity to self-renew and differentiate into phenotypically heterogeneous, aberrant progeny. The following sections give a limited overview of studies that have reported the anti-cancer effects of ITCs in both *in vivo* and *in vitro* model systems.

Lung Cancer

The nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is found in cigarettes and is a major pro-carcinogen linked to lung cancer formation [2]. The results of several studies have indicated certain ITCs can inhibit the formation of NNK induced lung cancer. These studies have led to a Phase II clinical trial (NCT00691132), to determine the effects of PEITC in participants who smoke only deuterated NNK cigarettes. The endpoint this study is to determine if PEITC consumption affects the urinary levels of NNK biomarkers in

current smokers. This research group will also determine if there are changes in cell proliferation or apoptotic bodies in lung biopsies taken after ingesting PEITC or placebo [10].

In animal models, SFN and PEITC have both shown to inhibit lung tumor formation and progression. SFN and PEITC both induced apoptosis in lung tissue of A/J mice in which cigarette carcinogens had induced a lung adenoma [2,10]. SFN and PEITC also inhibited the progression of adenomas to adenocarcinomas in the lung, implying that these treatments are useful in lung cancer chemoprevention [2,10]. In cell culture experiments, using various non-small-cell lung carcinoma (NSCLC) cell lines SFN, BITC, and PEITC have all been shown to inhibit cellular proliferation at low concentrations [28,35,49]. Additionally, both SFN and PEITC induced apoptosis in A549 lung cells after treatment with 20μM and 40μM of each ITC for 24 hours [49]. In several studies PEITC was shown to be a stronger inducer of apoptosis than SFN [2].

PEITC and BITC were reported to reduce migration and invasion of the highly metastatic NSCLC cell line, L9981 [35]. This study also demonstrated that BITC and PEITC inhibited phosphorylation of AKT, while also inhibiting activity of NF-κB and reducing metastasis related gene expression [35]. Additionally, Di Pasqua *et al.* (2010) showed that pretreatment with 10 μM of BITC or PEITC sensitized NCI-H596 NSCLC cells to cisplatin [28].

Breast Cancer

ITC consumption has been linked with a reduction in the risk of breast cancer for some time, and has recently been investigated as a breast cancer treatment option [17,21,40]. Currently, there are three clinical trials recruiting breast cancer patients, all of which are in Phase II. Two studies are to determin if the consumption of broccoli sprout extract (SFN) can alter

proliferation of breast tissue (NCT00982319 and NCT00843167). The study being completed by the Shannon lab in Oregon (NCT00843167) is focusing on histone deacetylase (HDAC) activity and apoptosis in women diagnosed with breast cancer, ductal carcinoma *in situ* and/or atypical hyperplasia. Additionally, a clinical trial being sponsored by the Sidney Kimmel Comprehensive Cancer Center (NCT00894712) is assessing the protective effects of topical SFN administration on radiation-induced dermatitis in women undergoing external-beam radiation therapy for breast cancer.

Several animal feeding studies have indicated that ITC treatment can inhibit breast cancer carcinogenesis. Recently it was published that BITC consumption could inhibit mammary carcinogenesis in MMTV-neu mice [34]. The results of this study suggested that a diet supplemented with 3mmol BITC/kg of chow could significantly decrease the number of Ki-67 positive cells and increase the number of apoptotic bodies in mammary tumors [40].

SFN, BITC, and PEITC are reported to inhibit cell growth and induce apoptosis in breast cancer cell lines, such as the MCF-7 cell line [11,49]. Additionally, BITC was reported to inhibit epithelial-mesenchymal transition (EMT) in the MDA-MB-231 human breast cancer cell line. This was also supported in xenografted human breast cancer cells [39]. Moreover, BITC was shown to inhibit hypoxia inducible factor (HIF1-α) expression and activity in MCF-7 cells by targeting the 4E binding protein 1 (4E-BP1) [15,17].

Studies that target cancer stem cells are currently of great interest, and SFN was found to decrease the cancer stem cell marker, aldehyde dehydrogenase-1 (ALDH-1), in MCF-7 and SUM159 human breast cancer cell lines [50]. Additionally, daily injections of SFN (50 mg/kg) for 2 weeks reduced the number of ALDH-1 positive cells by 50% in non-obese/severe combined immunodeficient mice with SUM159 xenograft tumors [36]. Treatment with SFN

also decreased the number of primary mammospheres, which are known to have cancer stem-like cell properties, by 8- to 125-fold in these two cell lines (MCF-7 and SUM159) [50].

Prostate Cancer

Epidemiological evidence is mixed when investigating the relationship between cruciferous vegetable consumption and prostate cancer. In eight case-control studies published since 1990, the results of four studies showed a significantly lower incidence of prostate cancer in men who consumed large amounts of cruciferous vegetable [1,16,21]. The other four studies indicated that there was no significant difference. Limiting the analysis to men who are positive for prostate specific antigen (PSA) appears to decrease some bias and show a stronger link between ITC consumption and the reduction of prostate cancer [16].

A current clinical trial (NCT01265953) is attempting to identify mechanisms by which SFN capsules can alter gene expression via epigenetic modifications in patients at risk for prostate cancer development. The investigators are particularly interested in studying SFN's effects on HDAC and DNA methylation in biopsies from men at risk for prostate cancer. ITCs are also reported to target prostate tumors, and there is a Phase II clinical trial (NCT01228084) which is recruiting patients with recurrent prostate cancer. This trial will determine if treatment with SFN can decrease PSA levels within 20 weeks of the SFN treatment.

In addition to the clinical trials, the goals of many *in vivo* and *in vitro* studies were to investigate ITC's ability to inhibit and target prostate cancer [1,14,24,30]. Xiao *et al.* (2010) recently published that PEITC can sensitize PC-3 and DU145 cells to docetaxel [51]. In the same study, intraperitoneally injected PEITC in combination with docetaxel was shown to up-regulate pro-apoptotic proteins (Bax and Bak) greater than either PEITC or docetaxel treatment

alone in PC-3 xenografts in male athymic mice [51]. Additionally, treatment with PEITC and docetaxel combined inhibited average tumor volume significantly more than either treatment alone. One mechanism proposed by which ITCs can exhibit antitumor effects on prostate cancer cells is via the JAK/STAT3/IL6 pathway [14,30,35,52]. The results of two independent studies have suggested that both SFN and PEITC can inhibit STAT3 activation and IL-6 production [15,32]. Inhibition of STAT3 activation is proposed to be one way in which ITCs produce pro-apoptotic effects [14]. Additionally, BITC treatments were found to inhibit cellular growth and induce G2/M cell cycle arrest in DU145 cells [30]. Treatment with BITC also stimulated apoptosis in DU145 cells through the release of apoptosis-inducing factor (AIF) and endonuclease G (endoG) from the mitochondria, and promoted caspase-3 activation [53].

Pancreatic Cancer

Currently, there are no clinical trials investigating the effects of ITCs on the prevention or treatment of pancreatic cancer. However, several animal models have suggested that BITC, PEITC, and SFN can inhibit pancreatic tumor formation. For example, in an animal model using Syrian Hamsters, PEITC was shown to inhibit the formation of N-nitrobis (2-oxypropyl) amine (BOP)-induced pancreatic tumors [54]. In cell culture, BITC and PEITC are reported to inhibit NF-kB activity, STAT3 activation, and induce reactive oxygen species [25,55,56]. Additionally, SFN alone or in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) significantly reduced the growth of pancreatic tumors that are rich in tumor initiating cells, or cancer stem cells, while not causing cytotoxic or adverse effects in normal pancreatic cancer cells [57]. Rausch *et al.* (2010) also suggested that SFN in combination with sorafenib

has a synergistic effect in targeting pancreatic cancer stem cells, by decreasing clonogenicity, spheroid formation, and ALDH1 activity [43].

Hematological Cancer

Literature indicates that PEITC has been of significant interest in treating various hematological cancers, such as Acute Myeloid Leukemia (AML) and multiple myeloma [2]. A Phase I clinical trial (NCT00968461) was scheduled to begin in January of 2012 by the MD Anderson Cancer Center. The objective of the study is to identify the highest tolerable dose of orally administered PEITC that can be given to patients who have lymphoproliferative disorders and have previously been treated with the drug fludarabine.

An *in vitro* study of great clinical interest demonstrated that primary Chronic Lymphocytic Leukemia (CLL) cells, both resistant and responsive to fludarabine, were highly sensitive to PEITC with an IC_{50} of 5.4 and 5.1 μmol/L, respectively [29]. However, normal lymphocytes did not show sensitivity to PEITC until 27 μmol/L [29]. In other cell culture experiments, PEITC treatments inhibit cellular growth and induce apoptosis in the U937, Jurkat, and HL-60 human leukemia cell lines [58].

SFN and PEITC treatments inhibited proliferation of primary human acute myeloid leukemia (AML) cells *in vivo* and *in vitro* [58,59]. In AML cell lines, both SFN and PEITC induced cleavage of PARP and caspases-3 and-9 in a concentration dependent manner [3,60]. PEITC treatments also inhibited NF-κB, activated the JNK pathway, and inhibited the AKT pathway in the U937 human leukemia cell line, indicating a mechanism through which PEITC can induce apoptosis [58].

Head and Neck Cancer

Studies investigating the effects of ITCs on Head and Neck Cancer are limited. A few epidemiologic and basic research studies have suggested that a diet rich in cruciferous vegetables may reduce the risk of developing primary head and neck tumors [61]. As with NSCLC, smoking is the biggest risk factor for head and neck squamous cell carcinoma (HNSCC) [62]. Numerous reports using either animal models and human subjects suggest that ITCs can reduce the activity of carcinogens found in cigarettes, such as NNK [54]. All the *in vivo* studies to date have looked at inhibition of lung carcinogenesis in response to cigarette carcinogens. Future studies need to investigate if ITCs can reduce the incidence of NNK induced HNSCC carcinogenesis in animal models.

However, as with the other cancers, BITC, PEITC and SFN inhibited proliferation of HNSCC cell lines *in vitro* [2,44,61]. BITC also induced caspase-3 and PARP cleavage in the UM-22B and 1483 HNSCC cell lines in a time dependent manner [63]. In addition, BITC induced rapid activation of p38 MAPK, as well as activation of p44/MAPK in these same cell lines [61,63]. Another HNSCC study showed that a combination of SFN and radiation might produce a synergistic effect in decreasing proliferation in four HNSCC cell lines [45]. This study also indicated that a combination of SFN and chemotherapy increased apoptosis of HNSCC cell lines greater than treatment with either SFN or radiation alone. Data generated in our laboratory also suggested that BITC pretreatment sensitizes the highly resistant HN12, HN30 and HN8 HNSCC cell lines to cisplatin [44]. Additionally, we observed that BITC appears to inhibit migration of the HN12 HNSCC cell line in a dose dependent manner (**Figure 1.3**).

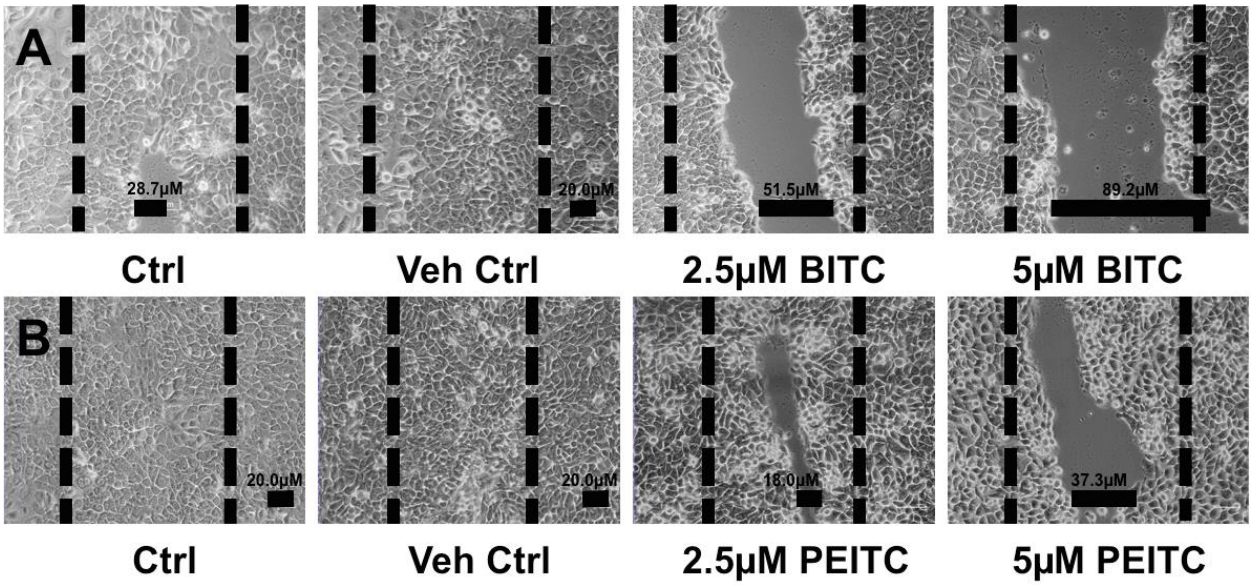

Figure 1.3 BITC and PEITC treatments inhibit wound healing in the HN12 cell line. **(A)** Inverted light microscope images of HN12 cells 24 hours after cells were treated with BITC (2.5-5µM) for 1-hour. **(B)** Inverted light microscope images of HN12 cells 24 hours after cells were treated with PEITC (2.5-5µM) for 1-hour. Dashed lines represent scratch size before treatment. Magnification 100X.

Conclusions

This chapter highlights the chemopreventive and chemotherapeutic potential of SFN, PEITC, and BITC. We discussed *in vitro* and *in vivo* data indicating that SFN, PEITC, and BITC treatments inhibit cellular proliferation, induce apoptosis, prevent metastasis, and inhibit angiogenesis of various cancers. We also provided evidence that ITCs can chemosensitize cancer cells. Interestingly, evidence also supports the notion that ITCs target cancer stem cells in breast, prostate, and pancreatic cancer. Furthermore, results from ITC clinical trials, while limited, suggest that the administration of SFN, BITC, or PEITC are safe treatment options and clinically relevant concentrations can be achieved.

The goal of this thesis is to further expand the knowledge of ITCs as a cancer treatment option. This chapter briefly highlighted the use of ITCs in HNSCC, and in following chapters we will present further results of studies to determine if BITC is a possible therapy option for HNSCC. Prior to presenting our findings on BITC treatment in HNSCC we will discuss why the need for new and alternative therapies for HNSCC is so important.

Chapter 2 **: Head and Neck Squamous Cell Carcinoma**

Epidemiology and Characterization

In the United States, head and neck cancer accounts for 3% of all malignancies and there are approximately 55,000 new cases each year and 12,000 related deaths [64]. Worldwide, head and neck cancer represents the sixth most common form of cancer, with over half a million new cases identified each year [65]. Two-thirds of head and neck cancer cases occur in industrialized nations and a predominant amount of patients are males in their 6^{th} and 7^{th} decade of life [66].

Head and neck cancer encompasses every type of tissue in the head and neck region, including the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx [66]. Head and neck squamous cell carcinoma (HNSCC) is the most prevalent form of head and neck cancer, and accounts for over 90% of all head and neck cancer cases [66]. HNSCC often develops from easily treatable dysplastic lesions; however most cases are not identified at this stage. More than 60% of HNSCC cases have with loco-regional (stage III) or distant metastasis (stage IV) at the time of diagnosis [67]. The five-year survival rate for localized HNSCC (stage I) and locally advanced with minimal lymph node involvement (stage II) is 80-90% [67]. However, the five-year survival rate is 20-50% for stage III and 10-30% for Stage IV [67].

A large portion of HNSCC cases are identified at stage III and IV, and not earlier stages due to the nature of the symptoms correlated with this type of cancer. The symptoms of HNSCC are often non-specific and can be attributed to other health conditions. For example, sore throat, nasal congestion, and hoarseness are common HNSCC symptoms and are often mistaken as the common cold, delaying proper diagnosis.

Major risk factors for developing HNSCC are tobacco use, alcohol consumption, and

infection with certain types of human papillomavirus (HPV) [66,68,69]. Additional risk factors include UV radiation, exposure to certain environmental toxins, and additional strains of viruses outside of the HPV spectrum, such as Epstein-Barr virus [66]. Certain risk factors are localized to specific regions. For example, in India there are a large number of people who use betel quids containing the aracea nut and this accounts for 50% of male HNSCC cases and 90% of female HNSCC cases [70]. However, the biggest universal risk factor for developing HNSCC is combining alcohol consumption with tobacco use, and this combination has been found to have a synergistic effect in inducing HNSCC [68].

Reports suggest that avoiding exposure to tobacco and alcohol products could reduce HNSCC by 90% [66]. A decrease in incidence of HNSCC in certain areas of the world is attributed to programs that educate the general public about the link between smoking, alcohol, and HNSCC. However, an increase in HPV infections in the Western world is diminishing the progress of reducing HNSCC [71]. While most cases of HNSCC are caused by tobacco and alcohol use, HPV-related head and neck cancers are on the rise [71]. HPV associated HNSCC account for a large portion of oropharynx cancers (cancer in the tonsils or the back of the tongue) [72]. In fact, it is reported that up to 80% of oropharyngeal cancers in the United States are due to infection with the HPV virus [71,73].

The identification of HPV as a risk factor for HNSCC is relatively new, and the presence of the HPV virus significantly alters a patient's treatment plan [71]. Research indicates that tumors initiated by HPV respond differently to treatments than tumors initiated by other causes, mainly because the genetic mutations and cellular pathways manipulated by HPV are different from the ones affected by other carcinogens [73].

Classification and Staging

There are eight types of HNSCC identified: conventional, verrucous, basaloid, papillary, spindle cell (sarcomatoid), acantholytic, adenosquamous, and cuniculatum [74]. Each of these variants can develop in any of the head and neck regions (oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx), except for cuniculatum (which is only identified in oral mucosa) [74]. HNSCC can be well-, moderately-, or poorly-differentiated and the histological grades used to classify HNSCC are found in **Table 2.1**. Most HNSCC cases are moderately to poorly-differentiated [74].

Table 2.1 Histological Grade used for HNSCC tumors.*

Histological Grade	
GX	Grade cannot be assessed
G1	Well differentiated
G2	Moderately Differentiated
G3	Poorly Differentiated
G4	Undifferentiated

*Information adapted from the National Cancer Institute (2014)

HNSCC may develop from precursor lesions (dysplasia), and in oral cancer these precursor lesions are called leukoplakia. Areas of keratosis in the oral cavity are typically white and can be classified into different grades (**Figure 2.1**). If untreated these pre-neoplastic lesions develop into squamocellular carcinoma (**Figure 2.2**).

HNSCC is also separated into different stages of diseases. The most common staging system used to grade head and neck cancers is the tumor node metastasis (TNM) classification

system. The TNM classification system is a cancer staging system that helps describe the extent and severity of a patient's cancer [75]. General TNM staging for head and neck cancer is described in **Table 2.2** and is further broken down in **Table 2.3**. In regards to HNSCC, T classifications are site specific and indicate the extent of the primary tumor, however there is overlap between the various head and neck regions when considering cervical node (N) classification [66].

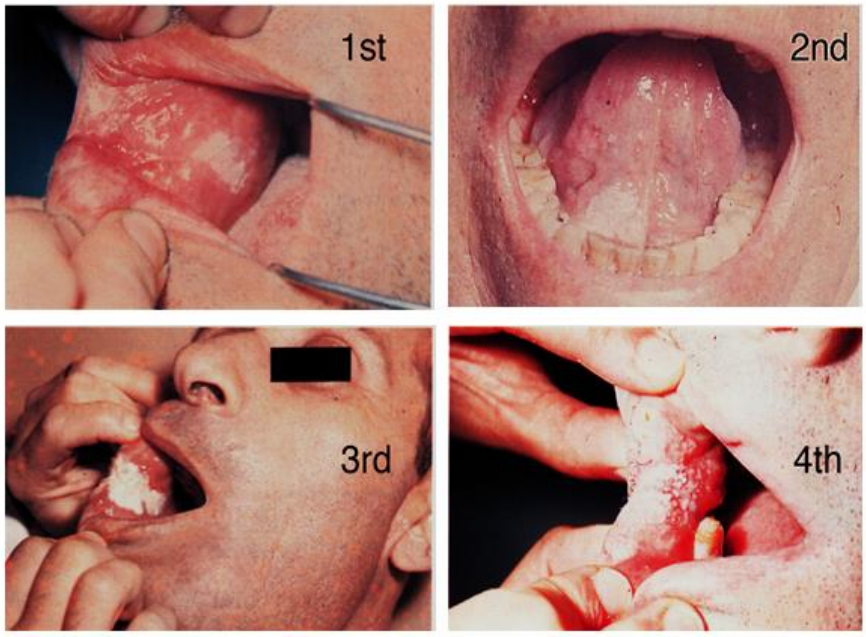

Figure 2.1 Preneoplastic lesions in the oral cavity.
The images depict four different stages of leukoplakia. Images are from the personal collection of P.P Claudio, M.D., Ph.D.

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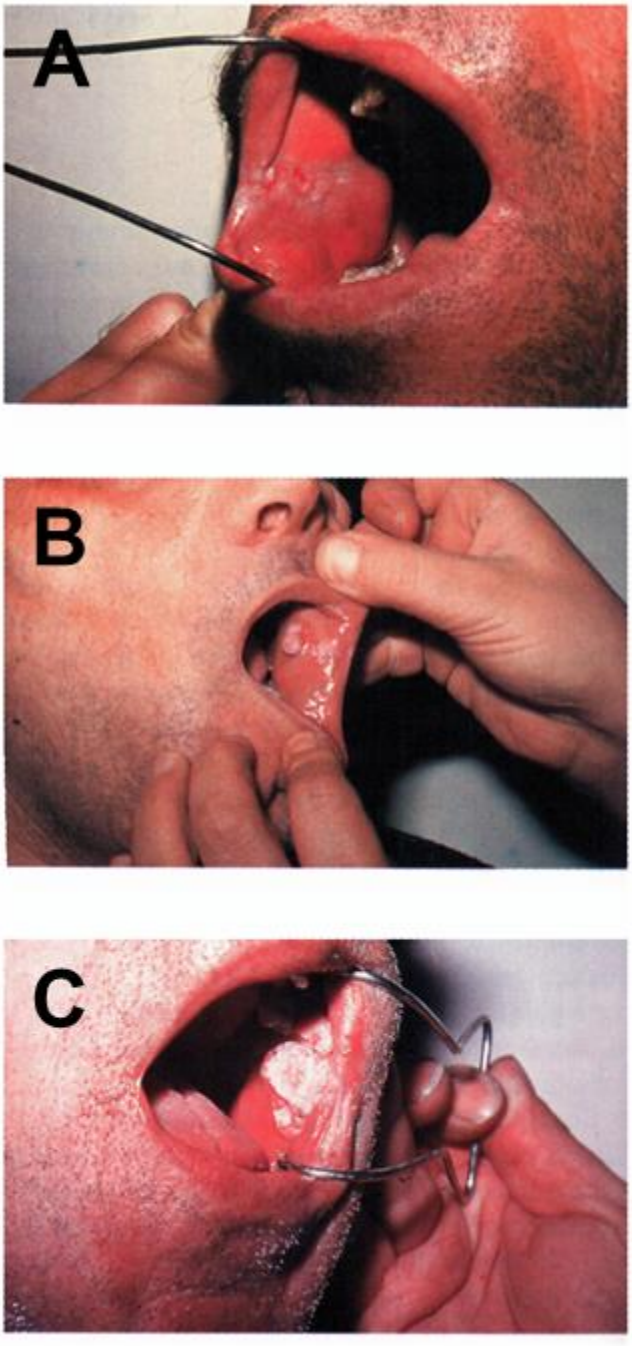

Figure 2.2 Presentation of preneoplastic lesions that have transformed into squamocellular carcinoma.
(A) Ulcerative carcinoma **(B)** Exophytic carcinoma. **(C)** Verrucous carcinoma. Images are from the personal collection of P.P Claudio, M.D., Ph.D.

Table 2.1 TNM Classification used in HNSCC. *

TNM Classification	
T	Describes the size of the primary tumor and the extent to which it has invaded nearby tissue
N	Describes whether regional lymph nodes are involved
M	Describes if the cancer has spread from one part of the body to another

*Information adapted from the National Cancer Institute (2014)

Table 2.2 General TNM staging of HNSCC. *

TNM Staging System		
	Staging	**Description**
Primary Tumor (T)	TX	Primary tumor cannot be evaluated
	T0	No evidence of primary tumor
	Tis	Carcinoma *in situ*
	T1-T4	Size and/or extent of the primary tumor
Regional Lymph Nodes (N)	NX	Regional lymph nodes cannot be evaluated
	N0	No regional lymph node involvement
	N1-N3	Degree of regional lymph node involvement
Distant Metastasis (M)	MX	Distant metastasis cannot be evaluated
	M0	No distant metastasis
	M1	Distant metastasis is present

*Information adapted from the National Cancer Institute (2014)

Diagnosis and Treatment

Computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) are techniques used, in addition to a physical exam, to diagnosis HNSCC [74]. If a mass is identified, a biopsy or fine needle aspiration are performed to evaluate whether the tumor is benign or malignant. These examination techniques are effective in identifying HNSCC, but unfortunately the initial symptoms of HNSCC are ambiguous and often overlooked [66]. Therefore, the first indication that a patient has head and neck cancer is usually after it has undergone metastasis to a local or regional lymph node [66]. Metastasis significantly impacts treatment and survival for HNSCC patients, and the most common metastatic sites are the lungs, bone, and liver. Pulmonary metastasis accounts for 60% of distant metastasis, while bone and liver metastasis account for 22% and 10% of distant metastasis, respectively [76]. HNSCC is also associated with second primary malignancies, which commonly occur in other areas of head and neck, as well as lung and esophagus [77].

After diagnosis, HNSCC treatment plans depend on the patient's age, location of the tumor, stage of the cancer, and general health of the patient [66,76]. A large portion of patients presenting with stage I or stage II HNSCC will be disease free after a single modality treatment [76]. However, a majority of patients present with advanced HNSCC and require multi-modality therapy to treat their disease [74,76]. Multi-modality therapy combines surgery, radiation, and/or chemotherapy, and a description of each of these treatments is available in **Table 2.3**. Multi-modality therapy is currently the best treatment approach for advanced disease due to the delicate nature of the head and neck region. This treatment approach helps minimize the concentration and dosage of any one therapy and also allows clinicians to preserve some function of affected tissue after surgery [76]. Minimizing dosage and exposure to anti-cancer therapy is important

because the oral cavity is highly susceptible to direct and indirect effects of chemotherapy and ionizing radiation.

An additional factor that has a significant impact on a patient's treatment is whether they test positive for HPV. Patients with HPV-positive oropharyngeal cancers are treated differently from their HPV negative counterparts [72]. Additionally, HPV-positive patients have a better prognosis and respond to less intense therapy, compared to HPV-negative patients. The current standard of treatment for HPV-positive tumors is cetuximab combined with radiation therapy.

Cetuximab is a monoclonal antibody (MAb) that represents an example of targeted therapy. Cetuximab inhibits the Epidermal Growth Factor Receptor (EGFR), which is often overexpressed in HNSCC. The goal of most targeted therapy is to identify biomarkers specific to HNSCC, and use this knowledge to create personalized therapies [78]. This new area of HNSCC research designs treatments that are to stimulate the immune system or control growth of cancer. In addition to the previously mentioned Cetuximab, targeted molecular therapies include other MAb, vaccines, and viruses, , [78,79]. These therapies are suggested to promote a more favorable outcome with less systemic side effects.

The poor outcome for advanced HNSCC opens the door for other alternative therapies. Nutritional compounds are new methods being explored in HNSCC therapy. These alternative interventions can be systemically or locally applied and can be used as adjuvants to traditional therapy. BITC is one form of adjuvant therapy being investigated for HNSCC and the effects of this compound on HNSCC will be highlighted in Chapter 3 and 4.

Table 2.3 HNSCC treatment options.*

HNSCC Treatment Options	
Therapy type	**Description of therapy**
Surgery	Allows for removal of primary tumor and some metastatic regions. Techniques include surgical resection and flap reconstruction. Common therapy at all stages.
Radiation therapy	Uses high-energy x-rays or others type of radiation sources to prevent cancer cells from growing. External and internal radiation is both used to treat HNSCC. External radiation uses a machine outside of the body to send radiation to tumor. Internal radiation uses radioactive substances sealed in needles, seeds, wires, or catheters that are placed directly into or near the cancer. Use of radiation depends on stage and grade of tumor.
Chemotherapy	Anti-cancer agents administered systemically or regionally to stop the growth of cancer cells, by killing them or preventing them from dividing. According to the National Cancer Institute (2014) there are seven chemo drugs approved by the FDA to treat head and neck cancer: Cisplatin, Carboplatin, Methotrexate, Fluorouracil, Bleomycin, Cetuximab, and Docetaxel. Use of chemotherapy depends on stage and grade of tumor.

*Information obtained from Up-to-Date (2014)

Pitfalls of Current Treatments

The most notable pitfall associated with HNSCC treatment is that the overall survival of patients has not improved over the last three decades. Currently, the National Cancer Institute (2014) suggests that the overall five-year survival rate for HNSCC is 40-50%, and this percentage is significantly lower if a patient develops metastasis or secondary tumor in the head and neck region. Around 60% of patients are diagnosed with locoregionally-advanced disease, and treatment of advanced HNSCC is aggressive, with significant acute and long-term effects [64]. Even with aggressive multi-modality therapy the five-year survival rate of locally advanced disease is only 30% [67]. Furthermore, the "standard of care chemotherapy" for advanced HNSCC disease only increases the five-year survival by 5% according to the Meta-Analysis for Chemotherapy of Head and Neck Cancer collaborative group [67].

On occasion, for loco-regionally advanced disease, a composite resection may be indicated for patients with primary tumors that entail extensive soft tissue disease around the mandible, thus requiring the need to sacrifice an intervening segment of mandible to accomplish an in-continuity resection of the primary tumor in conjunction with neck dissection. These particular operations are called "commando operation" or a composite resection that entail excision of the intraoral primary tumor along with a segment of the intervening mandible performed in conjunction with ipsilateral neck dissection as a monobloc surgical resection (**Figure 2.3**).

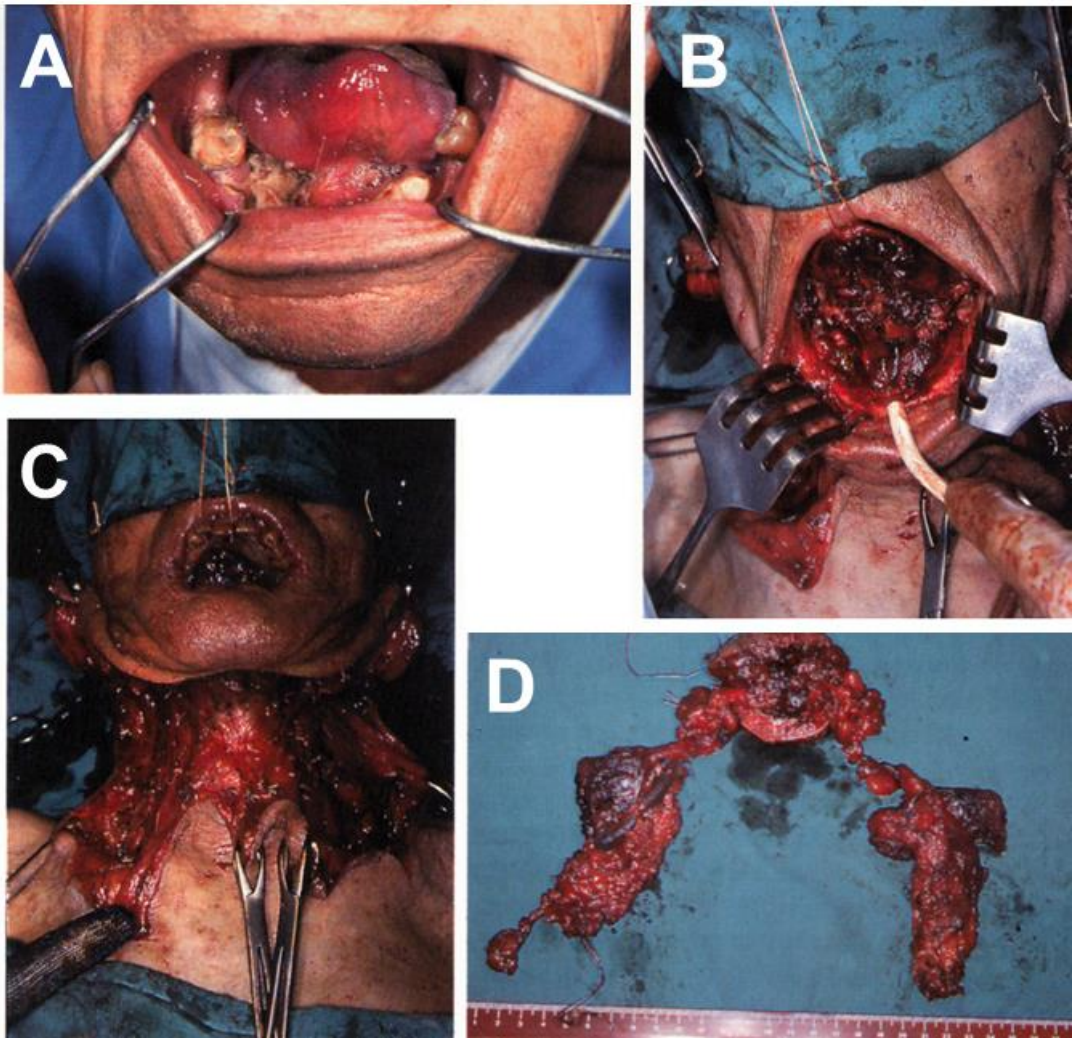

Figure 2.3 Presentation of locally advanced squamous cell carcinoma.
(A) Locally advanced oral pavement squamous cell carcinoma infiltrating the gingiva and the mandibular symphysis. (**B and C**) Patient undergoing a "commando operation" in conjunction with a bilateral neck dissection. **D)** Post operative specimen: oral pavement squamous cell carcinoma along with part of the tongue, a segment of the intervening mandible, and the bilateral neck lymph nodal dissection. Images are from the personal collection of P.P Claudio, M.D., Ph.D.

The aggressive nature of HNSCC and its delicate location makes it difficult to treat with a single modality therapy. A multi-modality approach is associated with the highest success rate for HNSCC, but often results in dramatic morphologic changes and increases in the inflammatory response [78]. The inflammatory changes can increase edema and fibrosis, which have significant impacts on a patient's health. Complications with eating, speaking, and breathing are additional effects commonly associated with both therapy and disease [78].

Chemotherapy and ionizing radiation also have a significant impact on the oral tissue and the gastrointestinal tract. The cell turnover in the oral mucosa and the lining of the gastrointestinal tract is rapid and leaves these areas very vulnerable to toxicity. The most common oral complications related to cancer therapies are mucositis, infection, salivary gland dysfunction, taste dysfunction, pain, and bleeding. Secondary complications can arise due to these side-effects and lead to dehydration, dysgeusia, and malnutrition [67]. The primary and secondary side-effects of treatment can inhibit optimal cancer therapy because a dose reduction and/or modification of the treatment schedule are needed.

Lastly, reoccurrence after treatment of HNSCC is high. If reoccurrence or distant metastasis occurs patients have a poor prognosis with a median survival rate of 6-10 months [80]. Recurrent tumors are also resistant or unresponsive to many of the initial therapies used treat HNSCC.

In conclusion, current therapy for stage I and stage II HNSCC is associated with high survival. However, patients with stage III and stage IV are provided a poor prognosis, and the therapy available has significant impacts on quality of life. Development of new therapies is needed to increase survival and improve health of patients with HNSCC. Investigation of

alternative treatment approaches, such as the administration of ITCs, could enhance the treatment of HNSCC without the detrimental side effects.

Molecular Mechanisms of HNSCC

This section will highlight the most frequently altered molecular pathways in HNSCC. Understanding the molecular and genetic alterations associated with HNSCC development will help uncover mechanisms involved in tumor formation and identify potential targets for improved therapy. Molecular techniques have already identified several tumor suppressor genes and proto-oncogenes linked to HNSCC development. Loss of heterozygosity (LOH) studies indicate that the earliest altercations appear in 3p (p16), 9p21 (CDKN2A1), and 17p13 (TP53). At later stages in tumor formation, deletions of 4q, 6p, 13q and 14q and amplication of 7p and 11q are reported [80,81].

Early events in HNSCC carcinogenesis are linked to inactivation of p16. Inactivation of p16 can be caused by LOH of 3p, homozygous deletion, point mutations, or promoter hypermethylation [81]. Loss of the p16 gene results in deregulation of both the p53 and pRB, and ultimately leads to uncontrolled proliferation [82]. Interestingly, instead of a p16 loss, HPV positive tumors have an overexpression of p16 in the nucleus and cytoplasm [80]. Therefore, p16 positivity is a reliable method for identifying HPV-positive tumors.

Around 70-80% of HNSCC cases have a loss of 9p21 (CDKN2A1) [81]. CDKN2A1 is a growth suppressor and novel cell cycle regulator. CDKN2A1 promotes cell cycle arrest by regulating the S phase of the cell cycle. TGFβ1 transcriptionally-induces CDK2AP1 expression, and this in turn mediates the growth inhibitory activity of TGFβ1 by interacting with Cdk2 and inhibiting pRb phosphorylation [80].

Approximately 60% of HNSCC's harbor a TP53 mutation [81]. p53 is described as the "guardian of the genome" and is an important regulator of the cell cycle. Studies report that alterations in p53 occur at a higher frequency in invasive carcinomas, compared to noninvasive carcinomas [82].

EGFR is overexpressed in most HNSCC cases [81]. EGFR activation regulates multiple pathways including: MAPK, Akt, ERK and the Jak/STAT pathway [81,83]. These pathways regulate cellular proliferation, apoptosis, and metastasis. Dysfunction of EGFR and its associated pathways occur in 80-90% of HNSCC cases [83].

Models used to study HNSCC

Cell Culture

HNSCC is a unique cancer because it includes a variety of different regions within the head and neck area. The diversity in tumor location can make designing *in vitro* models complicated, but many types of HNSCC develop from similar gene mutations. A more confounding factor than tumor location when selecting a cell culture model is the presence or absence of HPV, because HPV-positive cell lines respond differently to treatment compared to HPV-negative cell lines. HPV-negative tumors still account for around ~80% of all HNSCC cases. Therefore, in our studies we chose to focus on HPV-negative cell lines that represent the oropharangeal region. A model using the HN30 and HN12 cell lines was developed by George Yoo *et al.* (2000) [84]. This model was based on the observation that a p16 mutation is an early mutation in HNSCC development (HN30) and a p53 mutation occurs at a greater frequency at later stages and in invasive carcinomas (HN12) [84]. The cell lines used for in our studies are described in **Table 2.4.**

We also selected HN30, HN8, and HN12 because these cell lines represents three grades of migration potential. The HN30 cell line does not migrate, while HN8 and HN12 cell lines represent moderate and high migration, respectively. Selecting HN12 and HN8 cell lines for cell culture experiments have additional benefits that could be utilized in the future studies. HN12 and HN8 are synchronous lymph node metastases and primary tumor cell lines are available from the same patient.

Table 2.4 Characterization of HAK, HN12, HN30 and HN8 cell lines.

Cell Line*	Origin/Type	p53	p16	Tumorigenic	NFκB	Vimentin	Migration Capabilities
HAK	Normal Adult Keratinocytes	WT[a]	WT	No	Normal	-	None
HN30	Pharynx	WT	Del[c]	Yes	Constitutively active	+; only when grown in matrigel	Low
HN12	Lymph node metastasis from the tongue	Mut[b]	Del[c]	Yes	Constitutively active	+	High
HN8	Lymph node metastasis from primary oral tumor	Mut[b]	-	Yes	Constitutively active	Unknown	Moderate

*All cell lines are HPV negative; [a]WT=wild-type; [b]Mut=mutated; [c]del=deleted

Animal Models

There are several animal models used to study HNSCC. Models that use chemical carcinogens to induce tumor formation are ideal in HNSCC prevention studies. The most frequently used inducers are 9,10-dimethyl-1,2-benzanthracene (DMBA) and 4-nitroquinoline-1-oxide (4NQO) [81]. The carcinogen 4NQO can be administered through drinking water or via local application. Application of 4NQO replicates the human disease from preneoplasitc lesion to tumor. Additionally, this model activates oncogenes, including H-Ras (an EGFR), and is associated with spontaneous mutations in the tumor suppressor, p53, both of which are associated with HNSCC development [81].

No one particular xenograft model mimics HNSCC progression as is it observed in humans. Currently, the best animal model to mimic human disease progression is an orthotropic HNSCC model [85]. This model requires injection of exponentially growing HNSCC cells into the tongue of athymic nude mice. This model is associated with lymph node and regional

metastasis to the lung, however the percentage of mice that present with these forms of metastasis is inconsistent [85]. Plus, identifying lymph node metastasis is difficult. Furthermore, the time it takes to grow the tumors in the tongue is longer than most xenograft models. Also, these mice are reported to have problems eating and lose weight making feeding studies difficult.

In another model of HNSCC, exponentially growing HNSCC cells are being subcutaneously injected into the hindlimb of athymic mice [85]. It is easier to measure tumor formation with this model, than with the orthotropic model. This model is also acceptable for feeding or intravenous treatments. However, this model is not ideal to study metastasis.

Advancements are being made in techniques to monitor metastasis and this will greatly improve HNSCC animal models.

Chapter 3 **: Benzyl Isothiocyanate Inhibits HNSCC Cell Migration and Invasion, and Sensitizes HNSCC Cells to Cisplatin.**

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M. Allison Wolf (teter6@marshall.edu) [1,2] and Pier Paolo Claudio (claudiop@marshall.edu) [1,2,3,*]

[1] McKown Translational Genomic Research Institute, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, USA.

[2] Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755, USA.

[3] Department of Surgery, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25701, USA.

[*]Corresponding author:
Pier Paolo Claudio
McKown Translational Genomic Research Institute,
Joan C. Edwards School of Medicine,
Huntington, West Virginia, 25701
Phone: 304-696-3516
e-mail: claudiop@marshall.edu

Short title: BITC migration, invasion and sensitivity to CDDP.

ABSTRACT

Metastasis and chemoresistance represent two detrimental events that greatly hinder the outcome for those suffering with head and neck squamous cell carcinoma (HNSCC). Herein, we investigated benzyl isothiocyanate's (BITC) ability to inhibit HNSCC migration and invasion and enhance chemotherapy. Our data suggests that treatment with BITC: 1) induced significant reductions in the viability of multiple HNSCC cell lines tested (HN12, HN8, and HN30) after 24 and 48 hours, 2) decreased migration and invasion of the HN12 cells in a dose dependent manner, and 3) inhibited expression and altered localization of the epithelial-mesenchymal transition (EMT) marker, vimentin. We also observed that a pretreatment of BITC followed by treatment of cisplatin: 1) induced a greater decrease in HN12, HN30, and HN8 cell viability and proliferation compared to either treatment alone, and 2) significantly increased apoptosis when compared to either treatment alone. Taken together these data suggest that BITC has the capacity to inhibit processes involved in metastasis and enhance the effectiveness of chemotherapy. Consequently, the results indicate that further investigation, including *in vivo* studies, are warranted.

Keywords: Head and Neck Squamous Cell Carcinoma, isothiocyanate, BITC, migration, invasion, EMT, vimentin

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common form of cancer worldwide, and the 8th leading cause of cancer-related deaths [86]. Current treatment for HNSCC often entails a disfiguring and risky surgical operation, combined with chemotherapy and/or radiation therapy [87-89]. These treatment options are associated with numerous side effects that dramatically affect a patient's quality of life, and despite the aggressive treatment options the increase in overall survival of HNSCC has not improved in the past three decades [86,89-92]. The low survival rate is due, in part, to both loco-regional and distant metastasis, which occurs in 40-60 percent of HNSCC patients [89,92,93]. Additionally, the rate of HNSCC metastasis after recurrence is high, and relapse/recurrence is associated with heightened chemoresistance [92].

A common chemotherapeutic drug used for HNSCC is cisplatin, but the side effects associated with effective treatment doses can be severe. Additionally, one of the major obstacles in the therapeutic use of cisplatin is intrinsic or acquired resistance. Therefore, adjuvants therapies that enhance the efficacy of cisplatin and/or decrease the amount of cisplatin needed to achieve tumor response could improve patient outcome.

The acquisition of chemoresistance and the initiation of metastasis are complex multi-step processes. The use of natural products, such as isothiocyanates (ITCs), which are known to target many cellular pathways linked to both of these processes, provides a unique therapy option for HNSCC. ITCs are phytochemicals produced by several plant species, particularly cruciferous vegetables [6,49]. ITCs are a product of glucosinolate hydrolysis, which is initiated by an enzyme called myrosinase [2]. This enzyme is found spatially separated from glucosinolates in cruciferous vegetables and in our own human enteric microflora [2]. The

reactive group of ITCs, R-N=C=S, plays a significant role in ITCs involvement in numerous cellular pathways [4]. This functional group is known to target cysteine residues, which are often found in the catalytic site of many enzymes, and thereby can induce a wide range of effects inside a cell [7,8].

Benzyl isothiocyanate (BITC) is an ITC of particular interest in cancer therapy because of its ability to inhibit cell growth and induce apoptosis in several types of cancer cell lines, including HNSCC [2,25,94]. In addition to inhibiting cell growth and inducing apoptosis, BITC may play a role in inhibiting angiogenesis, epithelial-mesenchymal transition (EMT), and metastasis [13,40,95].

The present study builds on recent findings, which indicate that BITC may be able to inhibit metastasis and increase chemosensitivity. The evidence suggests that ITCs may prevent migration and invasion of several types of cancer cells, but the role of BITC in prevention of HNSCC migration and invasion has not been investigated. We elected to focus on BITC over other ITCs because our preliminary screenings suggested that the concentrations needed to elicit a response in HNSCC appear to be lower than other ITCs studied. Through the use of various *in vitro* studies we are reporting for the first time that BITC can inhibit migration and invasion of HNSCC cell lines. The potential use of BITC as an adjuvant treatment to inhibit metastasis, decrease markers associated with EMT, and enhance chemotherapy is a novel treatment approach.

MATERIALS AND METHODS

Materials

Benzyl isothiocyanate (99.5% pure) was purchased from LKT Laboratories, Inc. (St. Paul, MN). Stock solutions of BITC (100mM) were prepared in DMSO and diluted into growth medium such that the final concentration of DMSO did not exceed 0.02% (vol./vol.), a concentration that did not induce toxicity in HN12, HN30, HN8, and HAK cells. Cis-Diammineplatinum (II) dichloride (CDDP) was purchased from Sigma-Aldrich (St. Louis, MO). Stock concentrations of CDDP (1mg/1mL) were prepared in a 0.9% sterile saline solution.

Cell Culture and Reagents

The highly metastatic HNSCC cell line, HN12, and moderately metastatic HNSCC cell line, HN30, were a kind gift from Dr. George Yoo (Karmanos Cancer Center, Wayne State University, OH) [91]. The HN8 cell line was a gift from Dr. J. Silvio Gutkind (NIH, Bethesda, MD) [96]. The normal human adult keratinocyte cell line, HAK, was obtained from Zen-Bio, Inc. (Research Triangle Park, NC). Monolayer cultures of HN12, HN30 and HN8 were maintained in DMEM (HyClone, Thermo-Scientific) adjusted to contain 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) and supplemented with 1% (vol./vol.) penicillin-streptomycin (P/S) (Corning Cellgro, Manassas, VA). HAK cells were maintained in Adult Keratinocyte Growth Medium (KM-2) (Zen-Bio, Research Triangle Park, NC). Cells were grown in a humidified incubator at 37^{o}C and with 5% CO_2.

MTT Cell Viability Assay

HN12, HN8, and HN30 cells were seeded at an initial density of $5x10^3$ cells/well and HAK cells were seeded at an initial density of $15x10^3$ cells/well in 96-well tissue culture plates (Corning, Corning, NY) and allowed to settle overnight. The seeding density was selected so that all cell lines had a similar confluence after 24 hours. Cells were subsequently treated with 1.25-10μM BITC for 1-hour. After 1-hour plates were washed and media was replaced with fresh DMEM. The cell viability was determined after 24- and 48-hours using thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St. Louis, MO). Cells were incubated with dye for 2 hours, and then media was removed and replaced with DMSO. Color development in the plates was read at 590nm using the SpectraMax $M2^e$ plate reader (Molecular Devices, Sunnyvale, CA). The intensity of the color is correlated with the metabolic activity of living cells.

Wound Healing Assay

Cell migration was determined using wound healing assay. HN12 cells were cultured in DMEM (10% FBS, 1% Pen-Strep) in 6-well plates until 90% confluent, and then media was changed to DMEM with 0.05% FBS, 1% P/S overnight to synchronize the cells. A permanent line was drawn horizontally on the bottom of each well, and a plastic pipette tip was used to generate 3 vertical scratches per well. Cell debris was washed away with PBS and initial scratch sizes were determined with an inverted light microscope (Olympus IX51, Center Valley, PA) at 100X magnification. Six measurements were made per well, 1 below and 1 above the horizontal line for each scratch before treatment. Cells were treated with 2.5-5μM BITC for 1-hour at 37°C. DMSO, at the same concentration as in the BITC treated wells, was used for the vehicle control. After 1-hour plates were washed with PBS and treatment was replaced with DMEM

(10% FBS, 1% P/S). Wound healing was analyzed 24 hours after treatment. Images were taken at 100X magnification, as described above, and changes in cell migration were determined by calculating the percent of wound healing. Percent wound healing= ([$scatch_{t\text{-}0hr}$ - $scatch_{t\text{-}24hr}$]/$scatch_{t\text{-}0hr}$)*100. Experiments were repeated 3 times.

Invasion Assay

The effect of BITC on invasion of HN12 cells was determined using Invasion Chambers with 8μm pores (BD Biocoat, Franklin Lakes, NJ). Polycarbonate membranes on the bottom of the Boyden chamber inserts were rehydrated following manufacturer's instructions and 0.5mL of HNSCC cell suspension containing $5x10^4$ cells was added to each insert. Cells were allowed to attach for 4 hours prior to treatment in complete DMEM media (10% FBS, 1%P/S). After attachment the appropriate wells were treated for 1-hour with BITC (2.5-5μM) in serum free DMEM. Epidermal growth factor (EGF) was used at (10ng/1mL in serum free DMEM (0.5% BSA, 1% P/S)) was added to the bottom well in all wells, except for the negative control, as a chemoattractant. Media in all inserts was replaced after 1-hour with DMEM (0.5% BSA, 1% P/S). Analysis of cell invasion was performed 24 hours after beginning treatment. Media and cells were removed from the top of the matrigel following manufactures' instructions and cells were fixed with 100% methanol, washed with PBS, and stained with 0.1% Crystal Violet. Cells counts were performed and images taken using an Olympus IX51 inverted light microscope (Olympus, Center Valley, PA) at 400X magnification. Twenty fields of view were counted for each sample and averaged to determine the mean number of cells/field of view. Experiments were repeated a minimum of 3 times.

Western Blot Analysis

Vimentin and bcl-2 expression were analyzed 24 hours after BITC treatment in HNSCC cells. Cell pellets were lysed with RIPA buffer (1% NP-40, 0.1% SDS, 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.5% Sodium Deoxycholate, 1mM EDTA) for analysis. Densitometry was calculated using α-actin (SantaCruz, Santa Crutz, CA) as a loading control for all Western blots.

Vimentin antibody (AVIVA, San Diego, CA) was used at a 1:1000 dilution in a 5% milk/TBST buffer. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Rockland, Gilbertsville, PA) was used (1:10,000). The signal was developed with ECL Prime western blotting detection reagent (Amersham, Piscataway, NJ).

Bcl-2 antibody (SantaCruz, Santa Crutz, CA) was used at a 1:1000 dilution in a 5% milk/TBST buffer. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Rockland, Gilbertsville, PA) was used (1:5,000). The signal was developed with ECL Prime Western blotting detection reagent (Amersham, Piscataway, NJ).

Indirect Immunofluorescence

HN12 cells were seeded initially at a density of $4x10^4$ in Nunc Lab Tek II immunofluorescence chambers (Fisher Scientifics, Pittsburgh, PA). Cells were allowed to attach overnight before treatment with BITC (5-10μM) for 1-hour. Treatment media was then removed and replaced with complete DMEM (10%FBS, 1%P/S). Twenty-four hours after treatment, cells were fixed with 4% paraformaldehyde and permeabilized in PBS containing 1% BSA and 0.1% Triton X-100. Cells were blocked with PBS/1% BSA prior to staining. Vimentin (AVIVA, San Diego, CA) was diluted 1:400 with PBS containing 1% BSA and appropriate wells were incubated with primary antibody in a dark humidified chamber for 1-hour. Cells were then

washed and incubated with Alexa Fluor 488 (Invitrogen, Grand Island, NY) secondary antibody (1:200) in humid and dark conditions for 45 minutes. Slides were detached from immunofluorescence chambers and Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA) was added to slides before analysis. Images taken using an Olympus IX51 inverted microscope equipped with epifluorescence (Olympus, Center Valley, PA).

MTT Cell Viability/Chemosensitivity Assay

HN12, HN8, and HN30 cells were seeded at an initial density of $5x10^3$ cells/well in Corning 96-well tissue culture plates (Corning, NY) and allowed to settle overnight. Cells were then treated with 2.5-10μM BITC for 1-hour, 5-10μM of CDDP for 24 hours, or a 1-hour pretreatment of 2.5-10μM BITC followed by a treatment of 5-10μM of CDDP for 24 hours. Media was changed in all wells 24 hours after cisplatin treatment prior to the 48-hour analysis. Cell viability was determined 24 or 48 hours later as described above under MTT Cell Proliferation/Viability Assay methods. Results were performed in technical quadruplets with three biological replicates.

Trypan Blue Dead/Live Assay

HN12, HN8, and HN30 cells were seeded at an initial density of $3x10^5$ cells/well in Corning 6-well tissue culture plates (Corning, NY) and allowed to settle overnight. Cells were then treated with 5-10μM BITC for 1-hour, or 10μM of CDDP for 24 hours, or a 1-hour pretreatment with 5-10μM BITC followed by 10μM of CDDP for 24 hours. Cell counts were performed 24 and 48 hours after initiating CDDP treatment. Cells were trypsinized and washed

in PBS prior to cell count. Cells in each treatment group were mixed 1:1 with a trypan blue solution (0.4% trypan blue in PBS) and counted using a hemacytometer.

Annexin-V/PI Assay

HN12 and HN30 cells were seeded at an initial density of $2x10^6$ cells in 10cm Corning tissue culture dish (Corning, NY) and allowed to settle overnight. Cells were treated as described above in the Dead/Live assay methods. Treatment-induced cell death was determined by flow cytometry using an Annexin-V assay kit (eBiosciences, San Diego, CA). 24 and 48 hours after initiating CDDP treatment cells were collected and $1x10^6$ cells per group were subjected to a double staining with an Annexin-V-FITC antibody and Propidium Iodide following the manufacturer's instructions. Cells were analyzed using an Accuri C6 Flow Cytometer (BD Accuri, San Jose, CA). Early apoptosis was defined as cells positive for Annexin-V-FITC only. Late apoptosis was defined as cells positive for Annexin-V-FITC and Propidium Iodide (PI). Necrosis was defined as cells positive for PI only

Statistical Analysis

All experiments were performed at least three times as independent experiments. Statistical analyses were done with a multiple comparison test with appropriate post-hoc test. GraphPad Prism (La Jolla, CA) was used for all statistical analysis. A *p*-value of <0.05 was considered statistically significant.

The combination index of BITC and cisplatin combination was analyzed using the following equation: $CI=(C_{A,X}/IC_{X,A}) + (C_{B,X}/IC_{X,B})$ as described in Zhao *et al.* (2010) [97]. In this equation CI is the combination index; $C_{A,X}$ and $C_{B,X}$ are the concentration of drugs A and B

used in a combination that generates x% of the maximal combination effect; ICx is the drug concentration needed to produce x% of the maximal effect. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively.

RESULTS

BITC Decreased the Cell Viability of Three HNSCC Cell Lines

MTT assay results indicate that a 1-hour treatment of 10μM BITC significantly decreased the cell viability ($p \leq 0.05$) of the HN12, HN8 and HN30 cell lines after 24 and 48 hours (**Fig 3.1 A and B**). However, a 1-hour treatment of 2.5-5μM BITC did not significantly affect the cell viability of these HNSCC cell lines after 24 hours, and at 48 hours a significant decrease in cell viability was only observed in the HN30 cell line after a 5μM BITC treatment (**Figure 3.1 A and B**). Treatment of the normal keratinocyte cell line (HAK) with 2.5-10μM BITC did not decrease cell viability (**Figure 3.1 A and B**). Thus, BITC has selective toxicity for HNSCC cancer cells. A 1-hour treatment was selected for future experiments because the cell viability of HNSCC cells was not significantly different whether treated with 2.5-10μM BITC for 1, 16, or 24 hours (data not shown).

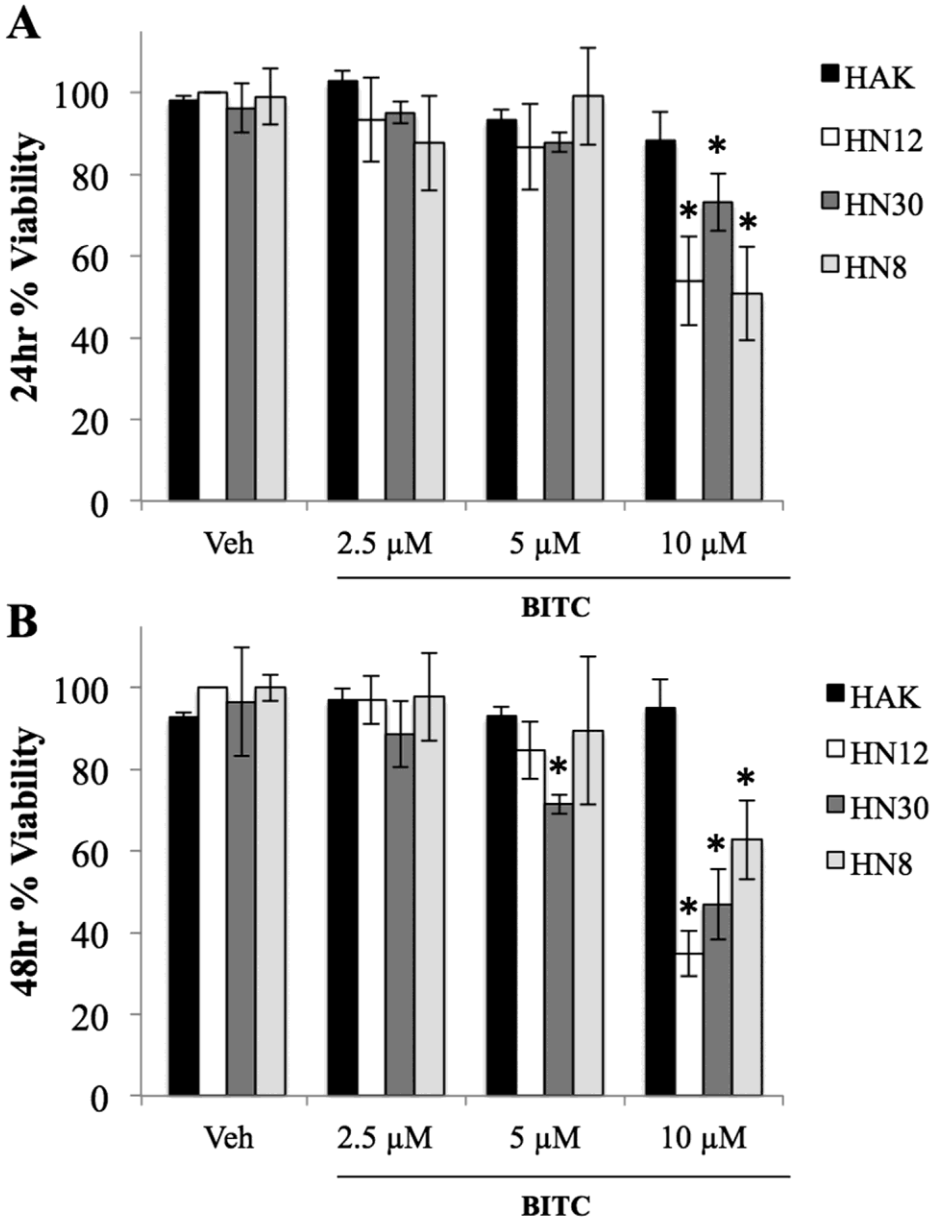

Figure 3.1 HNSCC percent viability measured by MTT assay following exposure to BITC treatments.
(A) Percent viability of HAK, HN12, HN30 and HN8 cells at 24 hours. **(B)** Percent viability of HAK, HN12, HN30 and HN8 cells at 48 hours. (*)indicates significant difference from respective cell line vehicle control. Percent viability was assessed by comparing treatment values to negative control. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$).

BITC Inhibited Migration and Invasion of HNSCC Cells

A wound-healing assay indicated that BITC inhibits migration of the highly metastatic HN12 cells in a dose dependent manner. After 24 hours, inhibition of cellular migration was observed in the highly metastatic HN12 cell line when using a 2.5μM BITC treatment, however a significant decrease in wound healing required 5μM BITC ($p \leq 0.05$) (**Figure 3.2 A and B**). Similar results were observed when using the HN8 cell line, but not the HN30 cell line (data not shown). Although, it should be noted that the HN30 cell line did not undergo "wound-healing" under control conditions.

The ability of BITC to inhibit the migration of HN12 cells prompted us to investigate the effect of BITC on invasion through Matrigel. **Figure 3.3** (panels A and B) depict that 1-hour treatment of BITC significantly inhibited the invasion of HN12 cells ($p \leq 0.05$). Compared to the vehicle control the average number of invading cells per field of view decreased by 52.34% after a 2.5μM BITC treatment and 90.96% after a 5μM BITC treatment ($p \leq 0.05$). Viability assays confirmed that the addition of the chemo-attractant, EGF, to the cells did not change the viability and proliferation of HNSCC cells after BITC treatment (data not shown). These results substantiate the wound-healing assay data and indicate that BITC targets both migration and invasion of certain HNSCC cell lines.

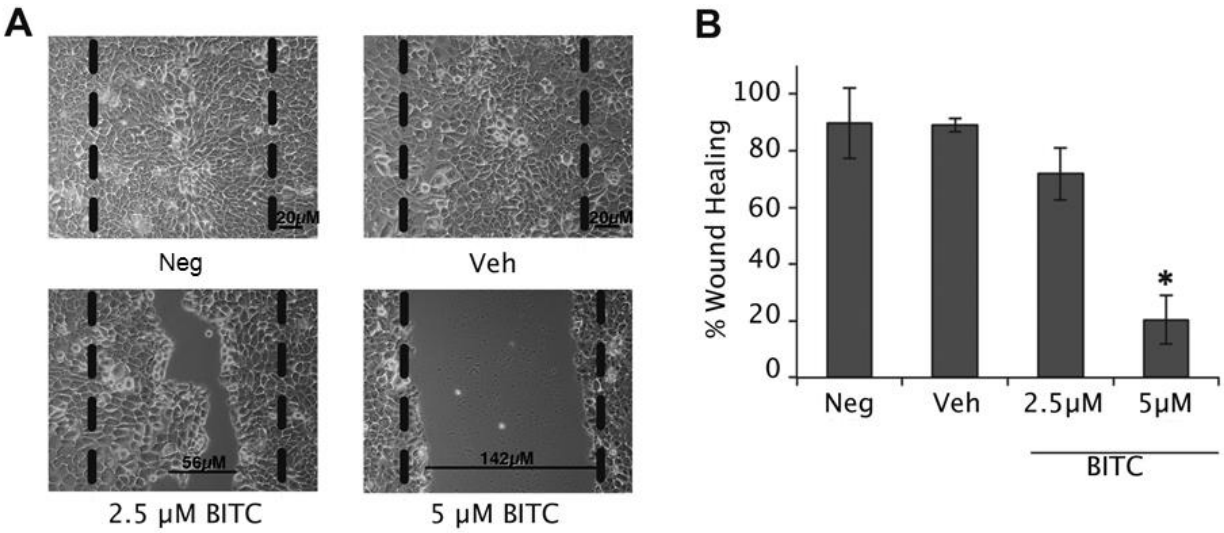

Figure 3.2 BITC inhibited wound-healing of the HN12 cell line after 24 hours.
(A) Inverted light microscope images of HN12 cells 24 hours after a 1-hour BITC treatment (2.5-5μM). Dashed lines represent scratch size before treatment. Vehicle control was DMSO. Magnification 100X. **(B)** Bar diagram represents the percent wound healing determined after 24 hours using wound size measurements. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$). Neg: negative control. Veh: vehicle control.

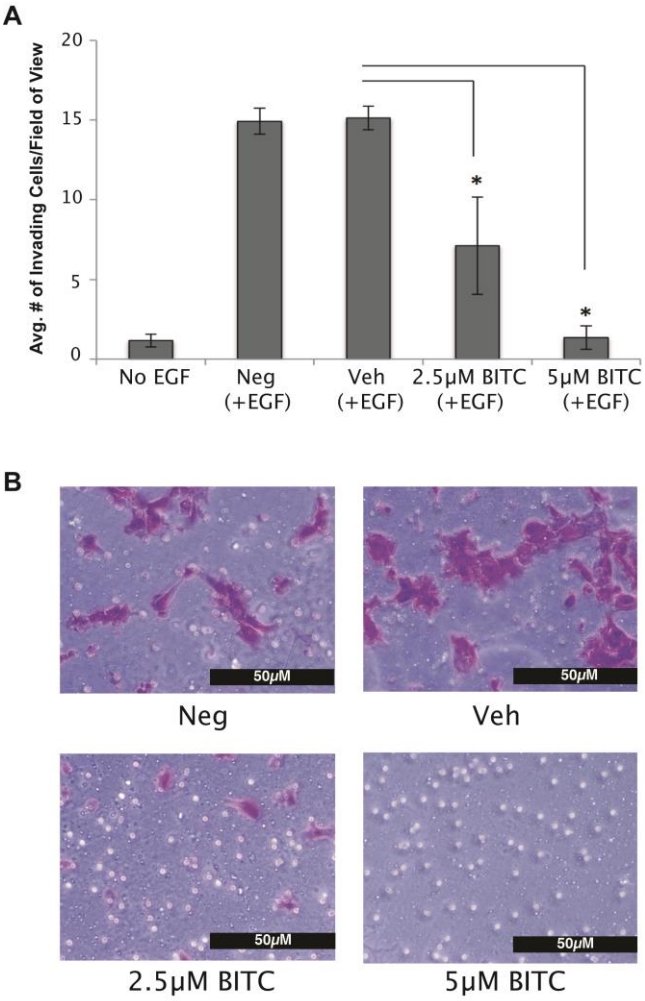

Figure 3.3 BITC treatment inhibited invasion of the HN12 cell line after 24 hours.
(A) Bar diagram represents the average number of invading cells/field of view counted at 24 hours following a 1-hour treatment of HN12 cells with BITC. EGF was used as chemoattractant. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$). **(B)** Inverted light microscope pictures of HN12 cells stained with crystal violet following BITC treatment. Magnification 200X. .Neg: negative control. Veh: vehicle control.

Vimentin Expression Decreased after BITC Treatment

Vimentin is an intermediate filament protein associated with EMT and HNSCC cell invasion [98,99]. We determined BITC treatment alters vimentin expression or localization. We observed that vimentin expression was inhibited 24 hours after a 1-hour BITC treatment (2.5-10μM), in a dose dependent manner (**Figure 3.4 A**). The localization of vimentin also appeared altered after BITC treatment. In the vehicle control vimentin was evenly dispersed, and was also observed in cellular projections. However, after BITC treatment these projections disappeared, and vimentin was observed as aggregates inside the cells. Our immunofluorescence results are supported by Western blot analysis showing a significant decrease in vimentin expression occurred after a 24 hour BITC treatment of 5 and 10μM ($p \leq 0.05$) (**Figure 3.4 B and C**). Although the treatment conditions changed for the Western blot analysis, cellular viability of HNSCC cells did not change whether treated with BITC for 1, 16, or 24 hours (data not shown).

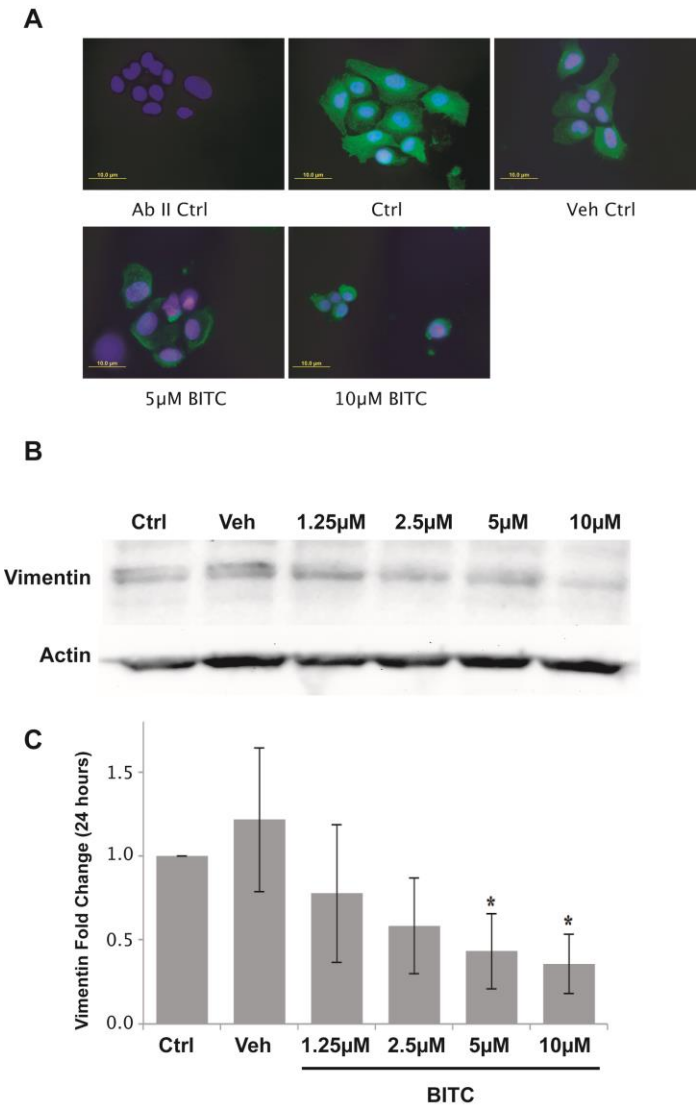

Figure 3.4 Vimentin expression in the HN12 cell line decreased following BITC treatment. **(A)** Representative immunofluorescence images of a vimentin immunostaining in HN12 cells acquired with an inverted epifluorescence microscope 24 hours after a 1-hour treatment with a range of concentrations of BITC (5-10µM). Anti-vimentin (1:400); AbII anti-rabbit Alexa Fluor 488 (1:200); DAPI to counterstain the nuclei. Magnification 400X. Bar size is 10µm. **(B)** Western blot analysis of vimentin expression in HN12 cells 24 hours after a 1-hour treatment with a range of concentrations of BITC (1.25-10µM). Actin was used to normalize the blot. **(C)** Densitometric analysis of vimentin and actin protein expression. Diagram represents the fold change of vimentin after 24 hours normalized to actin control. Ctrl: negative control. Veh: vehicle control. One-way ANOVA for multiple comparisons with Dunnett's Post-Hoc test (*$p \leq 0.05$).

Pretreatment with BITC Followed by Cisplatin Decreased HNSCC Cell Viability and Enhanced Cell Death

One of the major obstacles in the therapeutic use of platinum analogues is intrinsic or acquired resistance [100-103]. The high cisplatin resistance observed in our HNSCC cell lines prompted us to investigate whether BITC treatment of HNSCC cells enhances their response to CDDP.

A 1-hour pretreatment of BITC enhanced the effect of CDDP after 24 and 48 hours, compared to either BITC or CDDP treatment alone ($p \leq 0.05$) (**Figure 3.5 A-C**). The strongest decrease in cell viability was observed at 48 hours when the HNSSC cells were pretreated with 10µM BITC followed by 10µM CDDP treatment (Combination index CI= 0.52 to 0.93) ($p \leq 0.0001$) (**Figure 3.5 A-C**). Combination of 10µM BITC and 10µM cisplatin showed a synergistic effect in HN8 cells at 48 hours (CI= 0.73), but only additive effect at 24 hours (CI= 1.09).

A synergistic effect was also observed at 24 and 48 hours in HN30 (24 hours CI= 0.79; 48 hours CI= 0.52) and HN12 cells (24 hours CI= 0.86; 48 hours CI= 0.93) following a combination treatment of 10µM BITC and 10µM cisplatin (**Figure 3.5**).

The MTT assay assesses changes in cell viability, however the MTT assay does not differentiate between proliferation and/or cell death. Therefore, we used a trypan blue dead/live assay and an Annexin-V assay (apoptosis) to determine if BITC pretreatment increased cell death. The trypan blue dead/live assay (**Figure 3.6 A, B, D, E, G, and H**) indicated that cell death was significantly enhanced by BITC pretreatment followed by CDPP in all three HNSCC cells (HN8, HN12, and HN30), compared to treatment with either compound alone. However, the most dramatic increase in cell death was observed when HN30 cells were pretreated for 1-

hour with 10µM BITC followed by a 24-hour 10µM CDDP treatment, synergistic effect CI= 0.9; $p \leq 0.0001$) (**Figure 3.6 D**). These results were verified by numerical cell counts at the 24 and 48 hour time points of the different treatment groups (**Figure 3.6 C, F, and I**).

An Annexin-V assay showed a significant increase in early and late apoptosis after a pretreatment of 10µM BITC followed by 10µM CDPP in both the HN30 and HN12 cell lines after 24 hours ($p \leq 0.001$) (**Figure 3.7 A and B**). Additionally, the total percent of dead cells (early, late apoptosis, and necrosis) increased significantly when cells were pretreated for 1-hour with 10µM BITC followed by a 24-hour 10µM CDDP treatment ($p \leq 0.0001$).

Together the results of the MTT assay, dead/live assay, cell counts, and Annexin-V assay show that pretreatment of HNSCC cells with BITC followed by CDDP significantly increased HNSCC cell death relative to either agent used as a single therapeutic.

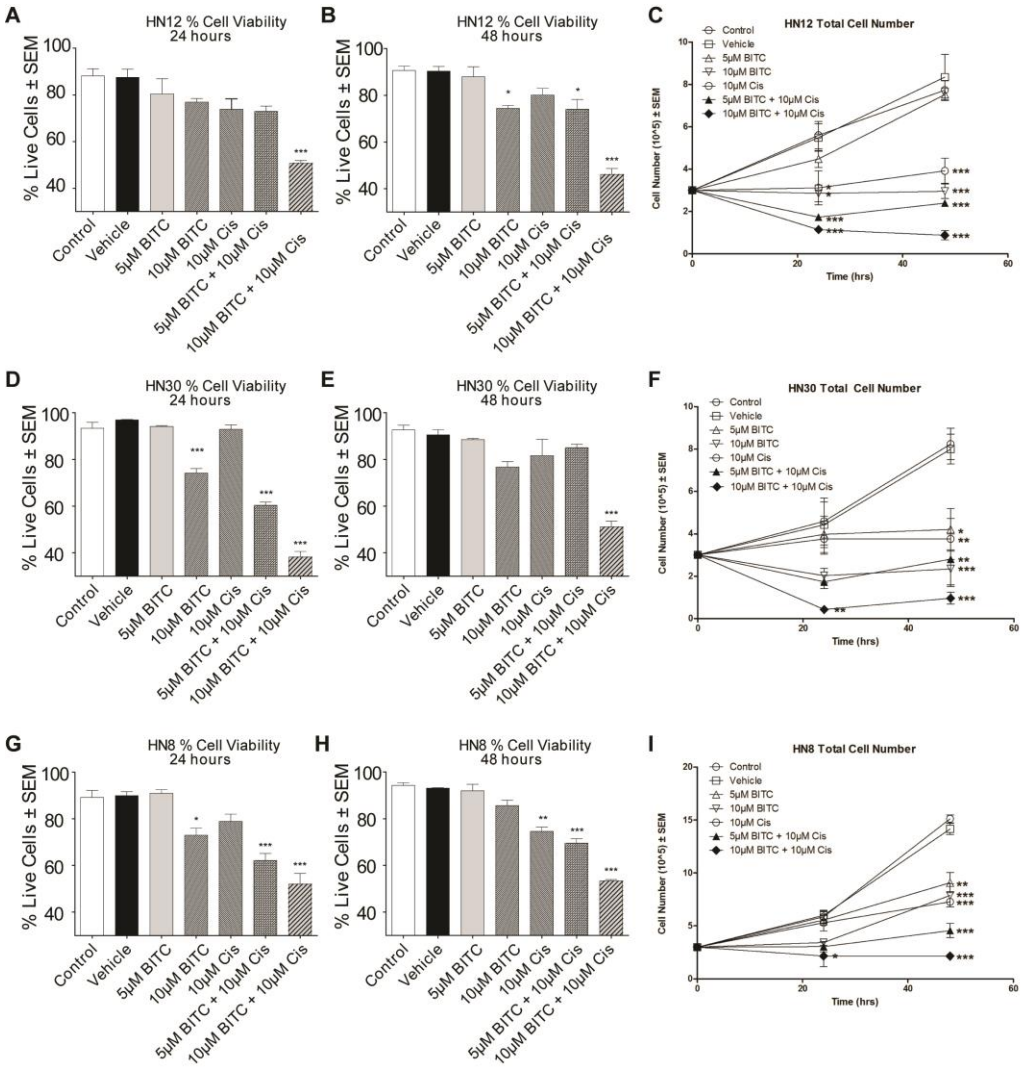

Figure 3.5 Dead/Live cell viability assay and cell counts of HNSCC cells following treatment with BITC, CDDP, or a pretreatment of BITC followed by CDDP.
(A-B) Diagrams represent the percent of HN12 cells that were alive after 24 (**A**) and 48 (**B**) hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. **(C)** Diagram depicts cellular proliferation of HN12 cell line 24 and 48 hours after a BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. **(D-E)** Diagrams represent the percent of HN30 cells that were alive after 24 (**D**) and 48 (**E**) hours following BITC, CDDP, or pretreatment of BITC followed by a CDDP treatment. (**F**) Diagram depicts cellular proliferation of HN30 cell line 24 and 48 hours after a 24 and 48 hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. **(G-H)** Diagrams represent the percent of HN8 cells that were alive after 24 (**G**) and 48 (**H**) hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. (**I**) Diagram depicts cellular proliferation of HN8 cell line 24 and 48 hours after a BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. Control: negative control. Vehicle: vehicle control. Cis: Cisplatin/CDDP. Error bars represent standard deviation. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

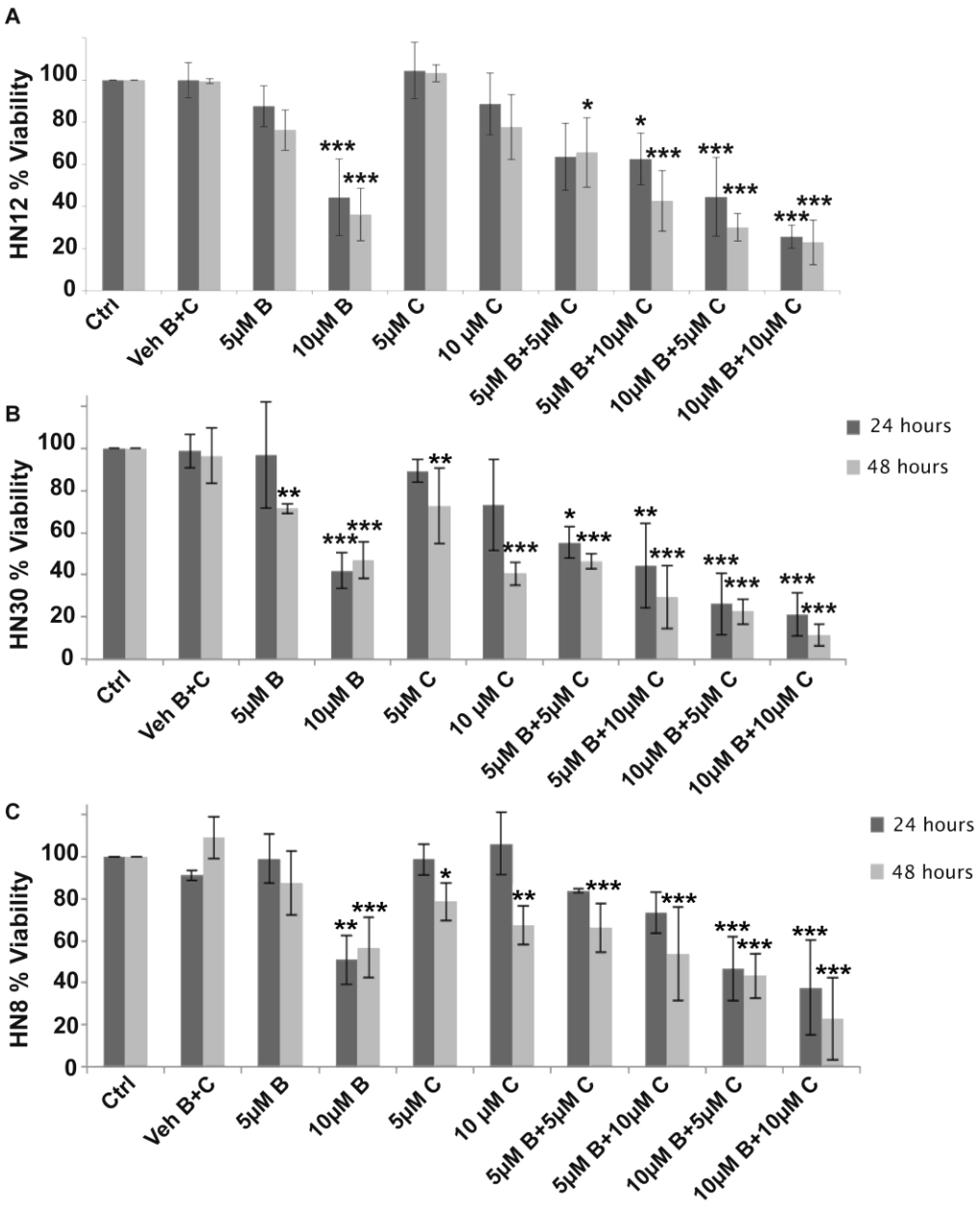

Figure 3.6 MTT viability assay of HNSCC cells following treatment with BITC, CDDP, or a combination of BITC followed by CDDP compared to controls.
(A) Diagram represents the percent of HN12 viable cells after 24 and 48 hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment (24 hours CI= 0.86; 48 hours CI= 0.93). **(B)** Diagram represents the percent of HN30 viable cells after 24 and 48 hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment (24 hours CI= 0.79; 48 hours CI= 0.52). (**C**) Diagram represents the percent of HN8 viable cells after 24 and 48 hours following BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment (24 hours CI= 1.09; 48 hours CI= 0.73). Dark grey bars indicate cell viability at 24 hours; light grey bars indicate cell viability at 48 hours. Ctrl: negative control. Veh B+C: vehicle control. B: BITC. C: Cisplatin/CDDP. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

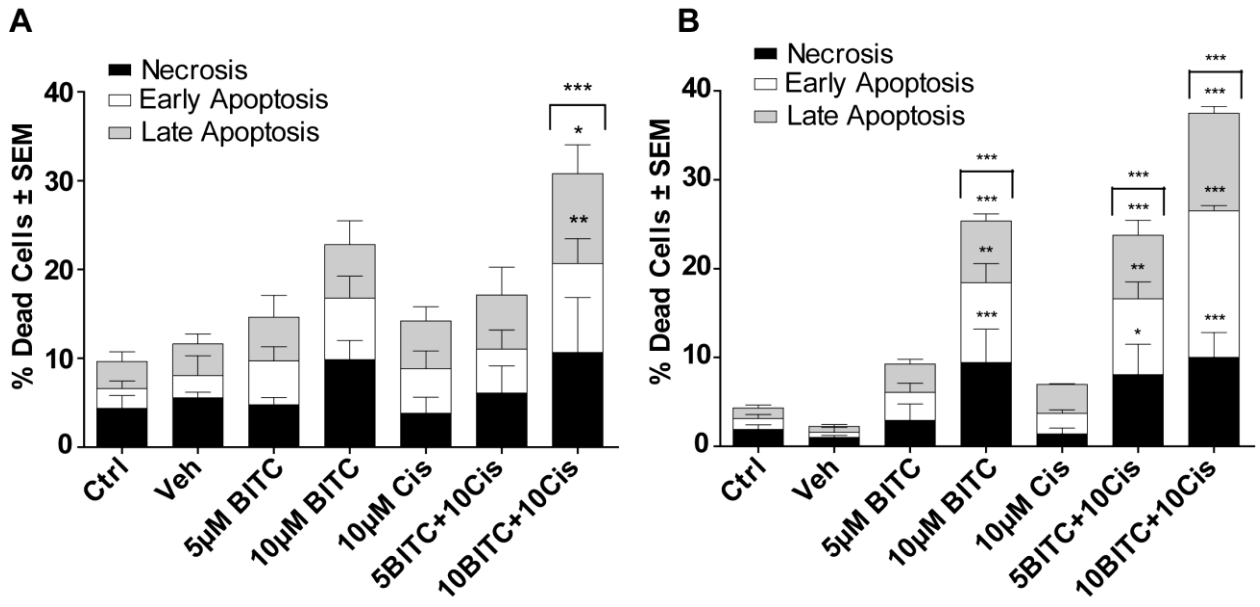

Figure 3.7 Annexin-V assay of HN12 and HN30 cells following treatment with BITC, CDDP, or a pretreatment of BITC followed by CDDP.
(A) Bar diagram represents the percent of HN12 cells that are in early/late apoptosis or necrosis 24 hours after a BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. **(B)** Bar diagram represents the percent of HN12 cells that are in early/late apoptosis or necrosis 24 hours after a BITC, CDDP, or a pretreatment of BITC followed by a CDDP treatment. Black bars represent necrosis, white bars early apoptosis, and grey bars late apoptosis. Error bars represent standard error of the mean. Ctrl: negative control. Veh: vehicle control. Cis: Cisplatin/CDDP. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

BITC Treatment Deceases the Expression of Anti-apoptotic Protein Bcl-2

Bcl-2 is an anti-apoptotic protein that is overexpressed in most cancer cells. Western blot analysis indicates that a 1-hour BITC treatment reduces the expression of bcl-2 in the HN12 cell line after 24 hours (**Figure 3.8 A and B**). A decrease in bcl-2 expression is observed after a 5μM BITC treatment, but the reduction in bcl-2 is not statistically significantly until after a 10μM BITC treatment ($p \leq 0.05$). A decrease in bcl-2 expression supports the annexin-V assay, and indicates that BITC treatments increase apoptosis in HNSCC cell lines.

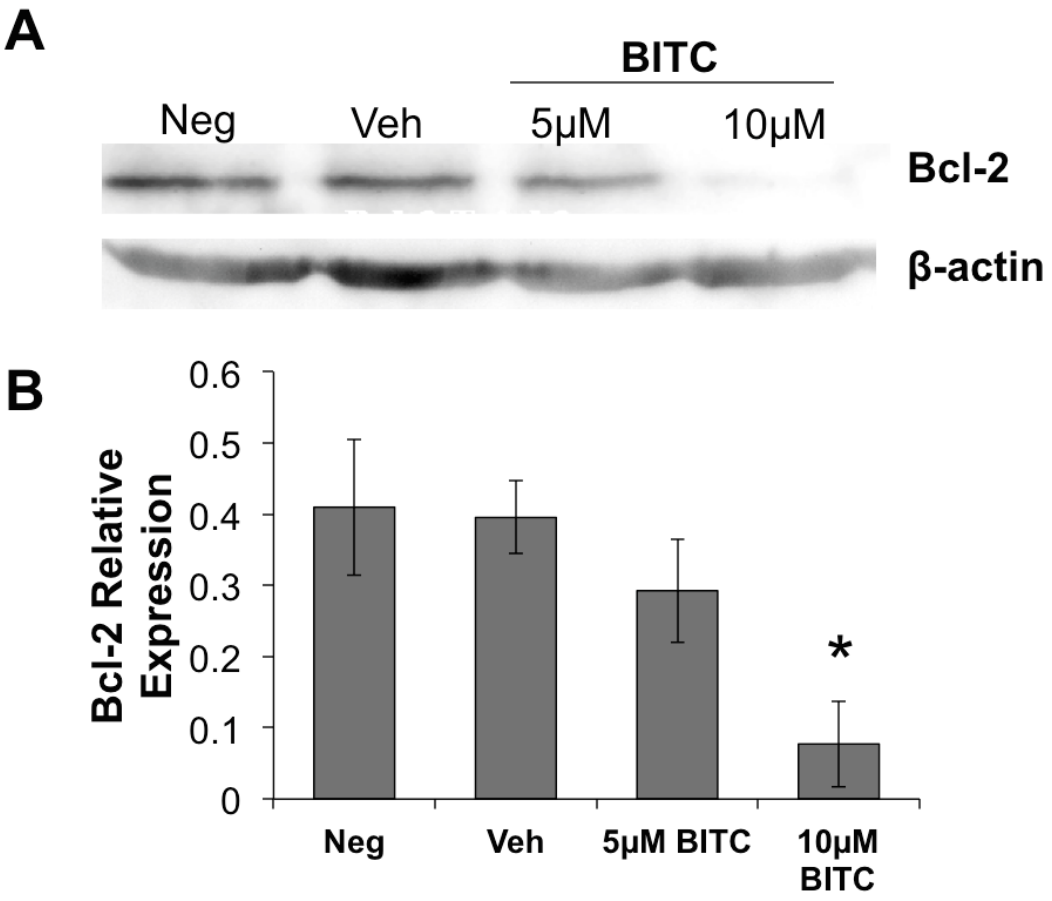

Figure 3.8 Bcl-2 Expression decreased in the HN12 cell line 24 hours after BITC treatment.
(A) Western blot analysis of bcl-2 expression in HN12 cells after a 1-hour treatment with 5 or 10μM BITC. Actin was used to normalize the blot. **(B)** Densitometric analysis of bcl-2 and actin protein expression. Diagram represents the relative expression of bcl-2 after 24 hours normalized to actin control. Neg: negative control. Veh B+C: vehicle control. B: BITC. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnett's Post-Hoc test (*$p \leq 0.05$).

DISCUSSION

The treatment for HNSCC often involves a disfiguring surgical operation and either prior or subsequent chemotherapy and/or radiation therapy [86,104]. These combined treatment modalities are often associated with side effects that reduce the patient's quality of life. HNSCC metastasis and chemoresistance are two of the leading reasons for this multimodal treatment approach. The inability to effectively target these events, whether separately or together, leads to a poor prognosis. Consequently, those who suffer from aggressive HNSCC face a debilitating disease, and need improved therapeutic options.

ITCs are natural compounds exhibiting potent anti-tumor effects in both cell culture and animal models [20,39,40,105]. In humans, ITCs are safe at clinically relevant concentrations and have high oral bioavailability, making them promising adjuvant therapy tools for the treatment of cancer [2,5,105]. Here, we show that BITC inhibits HNSCC cell migration and invasion, as well as sensitizes HNSCC cells to the chemotherapeutic drug cisplatin at clinically relevant concentrations [39,95]. Additionally, BITC decreased the expression of vimentin, a marker associated with EMT, in the HN12 cell line.

Tumors are not homogenous and may have different aberrant pathways that contribute to maintenance of cancer phenotype. The type of mutations present in different cancer cell could play a significant role on the different pathways and molecular targets of BITC. Since BITC's targets appear to be multifactorial we decided to focus primarily on the end points of cell death, proliferation, migration and invasion. Collectively our results illustrate that the effects of BITC, in regards to migration/invasion and chemoenhancement (greater anti-neoplastic effect than the sum of BITC or CDDP treatment alone) are not cell line specific. Importantly, BITC selectively targeted the viability of HNSCC cells, but not normal keratinocytes (**Figure 3.1**).

Vimentin expression in epithelial cells is a marker for epithelial-mesenchymal transition (EMT). Vimentin's overexpression in HNSCC correlates well with accelerated tumor growth, invasion, and poor prognosis [106]. Therefore, vimentin serves as an attractive potential target for HNSCC therapy. Many EMT markers are correlated with poor HNSCC prognosis, but few therapies have been shown to actually regulate the expression of these markers. Additionally, EMT, directly or indirectly links HNSCC metastasis and chemoresistance. Our findings that BITC treatment decreases vimentin expression in HNSCC cells suggest these cells are less likely to undergo EMT following BITC exposure indicating a unique property of this phytochemical that could be exploited for therapy.

Despite significant improvements in treatment modalities, long-term survival rates in patients with advanced-stage HNSCC have not increased significantly in the past 30 years. Radiation and chemotherapy are nonselective and can cause damage to normal tissue. Cisplatin is one example of a non-selective drug commonly used to treat HNSCC. This drug is associated with many detrimental side effects. Additionally, intrinsic or acquired resistance to platinum analogues is a major obstacle in HNSCC therapy. The results of our MTT assay, dead/live assay, cell counts, and Annexin-V assay support the use of BITC to either counteract cisplatin resistance or enhance its activity (**Figure 3.5 and Figure 3.6**). The concentrations selected for these studies mimic the peak plasma concentration of cisplatin [107,108].

In conclusion, our results show that BITC targets cell viability and reduces the amount of migration and invasion of HNSCC cells, but not of normal keratinocytes. The inhibition of migration and invasion we observed may be due to the ability of BITC to target key players involved in EMT, such as vimentin. Additionally, pretreatment with BITC chemosensitized the HNSCC cells to cisplatin by decreasing cell viability and increasing cell death suggesting that

BITC could be a novel adjuvant therapy for patients with aggressive HNSCC. These initial findings warrant future *in vivo* animal studies to ensure that BITC can reach tumor cells in adequate concentrations to induce xenograft or organotypic human HNSCC tumors to undergo the changes in viability and drug sensitivity that we have documented in cell culture.

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Chapter 4 **: Benzyl Isothiocyanate Induced ROS Regulates HNSCC Cell Migration and Viability**

ABSTRACT

In comparison to non-malignant cells, cancerous cells tend to display elevated levels of basal reactive oxygen species (ROS). As a consequence of the elevated levels of basal ROS, cancer cells are highly dependent on antioxidant defense systems. Therapeutic agents that abrogate or compromise the antioxidant system can leave cancer cells particularly vulnerable to the cytotoxic effects of ROS. Head and neck squamous carcinoma (HNSCC) cells are sensitive to treatments that alter the antioxidant system and induce ROS. Isothiocyanates (ITCs) are known inducers of ROS. Using a dicholorofluorescein (DCF) assay, as a measure of intracellular ROS, we demonstrate that the ITC, benzyl isothiocyanate (BITC), significantly increased ROS production in the HN12 and HN30 HNSCC cell lines. The increase in ROS was attenuated by the addition of antioxidants, *N*-acetyl-L-cysteine (NAC) and catalase. BITC, at concentrations that did not affect HNSCC cell viability, prevented wound healing in the HN12 cell line. The addition of either NAC or catalase reversed the wound healing inhibitory effect of BITC. NAC or catalase also prevented BITC-induced cytotoxicity in the HN12 and HN30 cell lines. Additionally, NAC reduced the cytotoxic effects of the combinatorial treatment of BITC and cisplatin. Collectively, these results suggest that ROS are important components in BITCs ability to induce cytotoxicity and inhibit migration in HNSCC.

Keywords: Head and Neck Squamous Cell Carcinoma, isothiocyanate, BITC, migration, reactive oxygen species, N-acetyl-L-cysteine, catalase

INTRODUCTION

We have previously shown that benzyl isothiocyanate (BITC) treatment inhibits migration and invasion and enhances the effects of the chemotherapeutic drug, cisplatin, in HNSCC cell lines [44]. However, the mechanism through which BITC elicits its effects on HNSCC cells remains unclear. In the present study, we investigated whether reactive oxygen species (ROS) induction is a possible mechanism by which BITC can prevent migration and add to the effectiveness of chemotherapy.

Malignant transformation often leads to an altered antioxidant system that promotes cancer cell survival. Additionally, pathways effected by these alterations lead to resistance from anti-cancer agents [44]. Manipulating the antioxidant system by altering the levels of glutathione (GSH) and/or ROS can provide a biochemical basis to selectively kill cancer cells, while leaving normal cells unaffected. Normal cells have low levels of basal ROS and the antioxidant system is able to tolerate inductions of ROS [23,98,109]. However, in cancer cells, an increase in basal ROS generation renders these cells highly dependent on the antioxidant system and agents that abrogate or compromise this system can leave the cancer cells vulnerable [109,110]. Consequently, anti-cancer agents that manipulate the redox balance are effective in treating a variety of cancers, including HNSCC [23,44,45,109,111].

Inducers of ROS are effective in triggering apoptosis in HNSCC cells thereby indicating that agents like isothiocyanates (ITCs), which are shown to increase oxidative stress, are ideal candidates for HNSCC therapy. However, ROS are still regarded as key meditators in numerous cellular pathways that lead to growth and survival, warranting a proper investigation in ROS inducing anti-cancer agents [23]. Traditionally, the research on ROS-inducing anti-neoplastic

drugs focused on whether the treatment kills cancer cells, and little attention has gone in to understanding how ROS inducers may effect migration and invasion [23,110]. The conundrum of ROS therapy is that ROS inducing agents are strongly associated with increased migration and invasion of cancer cells [112,113]. However, a study by Luanpitpong *et al.* (2010) showed that in HN460 lung cancer cells certain ROS (OH•) increased cell migration while others ($O2^{\cdot -}$ and H_2O_2) decreased cell migration. This same study indicated that catalase, which is a H_2O_2 scavenger, increased migration [114].

Results from our lab using HNSCC cells support the findings of Luanpitpong *et al.* (2010) and suggest that the type of ROS induced may be important in modulating HNSCC cell migration [114]. Here we show that BITC treatments significantly induced ROS and suppressed cell migration in HNSCC cell lines and that by modulating BITC induced ROS levels with either *N*-acetyl-L-cysteine (NAC) or catalase we restored HNSCC cell migration. In regards to decreased cell viability in HNSCC cell lines, we have shown that NAC inhibited the additive effects of BITC pretreatment followed by cisplatin.

In conclusion, BITC targets many molecular pathways in the cell that alter migration, invasion, and cell death. Here, we demonstrate that ROS play an important role in the ability of BITC to alter migration and invasion and induce cell death.

MATERIALS AND METHODS

Materials

Benzyl isothiocyanate (99.5% pure) was purchased from LKT Laboratories, Inc. (St. Paul, MN). Stock solutions of BITC (100mM) were prepared in DMSO and diluted into growth medium such that the final concentration of DMSO did not exceed 0.01% (vol./vol.), a concentration that did not induce toxicity in HN12 and HN30 cells. 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Grand Island, NY). Stock solutions of H_2DCFDA (50mM) were prepared in DMSO in the dark and diluted into serum free growth medium. *N*-Acetyl-L-cysteine (NAC) and Catalase from bovine liver were both purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of NAC (100mg/mL) were prepared in sterile Milli-Q water and diluted into serum free growth medium. Catalase treatments (2,500U/mL) were prepared in serum free growth medium. Cis-Diammineplatinum (II) dichloride (CDDP) was purchased from Sigma-Aldrich (St. Louis, MO). Stock concentrations of CDDP (1mg/1mL) were prepared in a 0.9% sterile saline solution.

Cell Culture and Reagents

The highly metastatic HNSCC cell line, HN12, and moderately metastatic HNSCC cell line, HN30, were a kind gift from Dr. George Yoo (Karmanos Cancer Center, Wayne State University, OH) [44,91]. Monolayer cultures of HN12 and HN30 were maintained in Dulbecco's Modified Eagles Medium (DMEM) (HyClone, Thermo-Scientific) adjusted to contain 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) and

supplemented with 1% (vol./vol.) penicillin-streptomycin (P/S) (Corning Cellgro, Manassas, VA). Cells were grown in a humidified incubator at 37°C and with 5% CO_2.

Intracellular ROS Generation

Cells were seeded in 6-well plates ($3x10^5$ cells/well) 24 hours prior to treatment. Levels of intracellular ROS were determined using 5-(and-6-) chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Eugene, OR). Cells were washed twice with 1X Dulbecco's PBS (PBS) (GIBCO, Invitrogen) and incubated for 5 minutes in the presence or absence of 2.5μM H_2DCFDA in serum free DMEM. Following a 5 minute incubation, cells were washed three times with 1X PBS and treated with BITC (1.25-10μM), 5mM NAC, 2,500U catalase, or a combination of BITC and NAC or BITC and catalase for 1 hour. After treatment cells were washed two times with 1X PBS and then incubated with StemPro® Accutase® (Life Technologies, Grand Island, NY) for 5 minutes. H_2O_2 (10μM) was used as positive control. Cells were collected and transferred to 1.5mL microcentrifuge tubes and spun down at 400xg for 5 minutes. The supernatant was removed and cells were washed two times with 500μL PBS. Cells were re-suspended in 400μL of PBS and kept one ice and fluorescence was determined using an Accuri C6 Flow Cytometer (BD Accuri, San Jose, CA). Assays for intracellular ROS generation were performed in technical triplicates and biological triplicates.

Immunofluorescence

Cells were seeded and treated as described above in the Intracellular ROS Generation methods. After treatment cells were washed two times with 1X PBS. Wells were filled with 2

mL of PBS prior to microscopy. Images were taken using an Olympus IX51 inverted microscope equipped with epifluorescence (Olympus, Center Valley, PA).

NF-κBp50 activity assay

NF-κBp50 activity was evaluated in HNSCC cell lines 24 hours after a 1-hour BITC treatment using an NF-κB p50 Transcription Factor Kit (Pierce Biotechnology, Rockford, IL), according to manufacturer's protocol. The kit contains streptavidin-coated 96-well plates and an NF-κB biotinylated-consensus sequence. Only the active form of NF-κB will bind to the DNA consensus sequence. A wild type NF-κB competitor and a mutant NF-κB competitor were used to ensure the signal specificity of the assay. A TNFα activated HeLa cell nuclear extract was used as a positive control. HNSCC cell pellets were lysed after treatment with RIPA buffer (1% NP-40, 0.1% SDS, 50mM Tris-HCl pH 7.4, 150mM NaCl, 0.5% Sodium Deoxycholate, 1mM EDTA). Protein extracts containing 5µg of protein/well were added in triplicates to appropriate microplate wells. Luminescence resulting from a reaction with bound NF-κB was detected using a SpectraMax L plate reader (Molecular Devices, Sunnyvale, CA).

Co-treatment MTT Cell Viability Assay

HN12 and HN30 cells were seeded at an initial density of $5x10^3$ cells/well in 96-well tissue culture plates (Corning, Corning, NY) and allowed to settle overnight. Cells were treated with BITC (2.5-10µM), 5mM NAC, 2,500U/mL catalase, and a combination of BITC and NAC or BITC and catalase for 1-hour. All treatments were prepared in DMEM containing 0.05% FBS, 1% P/S. After 1-hour plates were washed and media was replaced with fresh DMEM containing 10% FBS, 1% P/S. The cell viability was determined after 24- and 48-hours using

thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St. Louis, MO). Cells were incubated with the dye for 2 hours, and then media was removed and replaced with DMSO. Color development in the plates, which is correlated with the metabolic activity of living cells, was read at 590nm using the SpectraMax M2^{e} plate reader (Molecular Devices, Sunnyvale, CA).

Pre-treatment MTT Cell Viability Assay

HN12 and HN30 cells were seeded as described above in Co-treatment MTT Cell Viability Assay methods. Cells were treated with 1) BITC (2.5-10μM) for 1-hour, 2) a 1-hour pretreatment of NAC followed by a 1-hour BITC (2.5-10μM) treatment, 3) NAC alone for 1-hour, or 4) appropriate vehicle control. All treatments were prepared in DMEM containing 0.05% FBS, 1% P/S. After 1-hour of treatment MTT assay was performed as described above in Co-treatment MTT Cell Viability Assay methods.

Wound-Healing Assay

Cell migration was determined using a wound-healing assay. HN12 cells were cultured in DMEM containing 10% FBS, and 1% Pen-Strep in 6-well plates until 90% confluent, and then media was changed to DMEM with 0.05% FBS and 1% P/S overnight to synchronize the cells. A line was drawn horizontally on the bottom of each well with a permanent marker, and a plastic pipette tip (p200) was used to generate 3 vertical scratches per well (90 degrees to the permanent line). Cell debris were washed away with PBS and initial scratch sizes were determined with an inverted light microscope (Olympus IX51, Center Valley, PA) at 100X magnification. Six measurements were made per well, 1 below and 1 above the horizontal line for each scratch before treatment. Cells were treated with 1) BITC (1.25 and 2.5μM), 2) 5mM NAC, 3)

2,500U/mL catalase, 4) a combination of BITC and NAC, or 5) BITC and catalase for 1 hour at 37^{o}C. DMSO, at the same concentration as in the BITC treated wells, was used for the vehicle control. After 1-hour, plates were washed with PBS and media was replaced with DMEM (10% FBS, 1% P/S). Wound healing was analyzed 24 hours after treatment. Images were taken at 100X magnification, as described above, and changes in cell migration were determined by calculating the percent of wound healing. Percent wound healing= (($scatch_{t\text{-}0hr}$ - $scatch_{t\text{-}24hr}$)/$scatch_{t\text{-}0hr}$)*100. Experiments were repeated 3 times.

MTT Cell Viability/Chemosensitivity Assay

HN12 and HN30 cells were seeded at an initial density of $5x10^3$ cells/well in Corning 96-well tissue culture plates (Corning, NY) and allowed to settle overnight. Cells were treated with 1) BITC (5μM); 2) NAC (5mM); 3) Cisplatin (10μM); 4) co-administration of BITC (5μM) and NAC (5mM); 5) co-administration of cisplatin (10μM) and NAC (5mM) 6) a 1-hour pretreatment of BITC (5μM) followed by a treatment of CDDP (10μM) for 24 hours; or 7) a 1-hour pretreatment of BITC (5μM) with NAC (5mM) followed by a treatment of CDDP (10μM) for 24 hours. DMSO was used for the vehicle control. Media was changed in all wells 24 hours after cisplatin treatment prior to the 48-hour analysis. Cell viability was determined 24- or 48-hours later as described above under MTT Cell Proliferation/Viability Assay methods. Results were performed in technical quadruplets with three biological replicates.

Statistical Analysis

All experiments were performed at least three times as independent experiments. Statistical analyses were done with a multiple comparison test with appropriate post-hoc test.

GraphPad Prism (La Jolla, CA) was used for all statistical analysis. A *p*-value of <0.05 was considered statistically significant.

RESULTS

BITC Increases ROS in HNSCC Cell Lines

A dichlorofluorescein (DCF) assay indicated that BITC dramatically increased the ROS production in the HN12 and HN30 cell lines ($p \leq 0.05$) (**Figure 4.1**). Immunofluorescence supported the DCF flow cytometry results and indicated that BITC treatment increased ROS in the HN12 cell line after 1-hour (**Figure 4.2**). The percentage of HN12 cells that fluoresced after a 1-hour BITC treatment (2.5-10 μM) was similar, but the intensity of the DCF fluorescence was concentration dependent (**Figure 4.2**).

We also observed that while the non-metastatic HN30 cell line showed a significant increase in ROS production after BITC treatment, the increase in ROS was over 3 folds greater in the highly metastatic HN12 cell line. Notably, while 2.5μM BITC induced significant elevations in ROS in HN12 and HN30, this concentration of BITC did not induce significant changes in cell viability after 24 or 48 hours

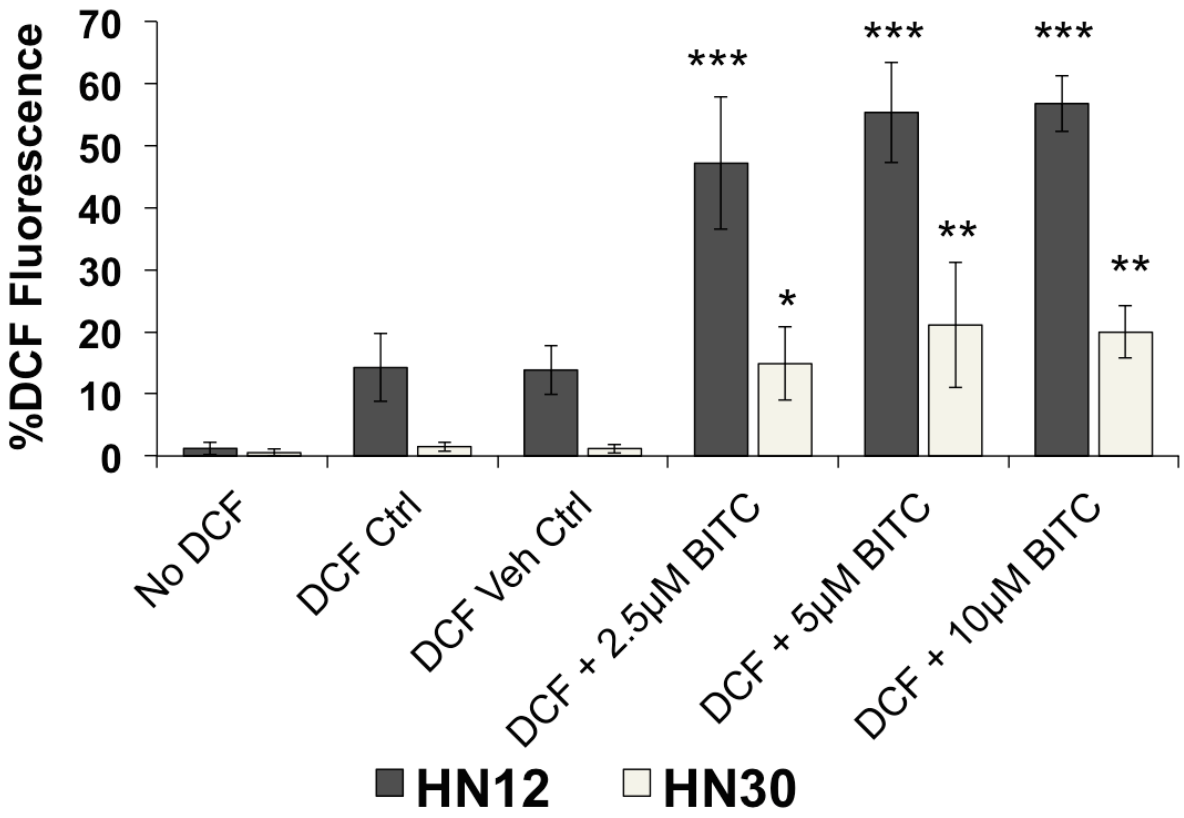

Figure 4.1 BITC treatment significantly increases ROS in HN12 and HN30 cell lines.
DCF assay was used to determine ROS production in HN12 and HN30 cell lines 1-hour after treatment with a range of concentrations of BITC (2.5-10µM). Dark grey bars represent HN12 cell line. Light grey bars represent HN30 cell line. Error bars represent standard deviation. One-Way ANOVA for Multiple Comparison with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

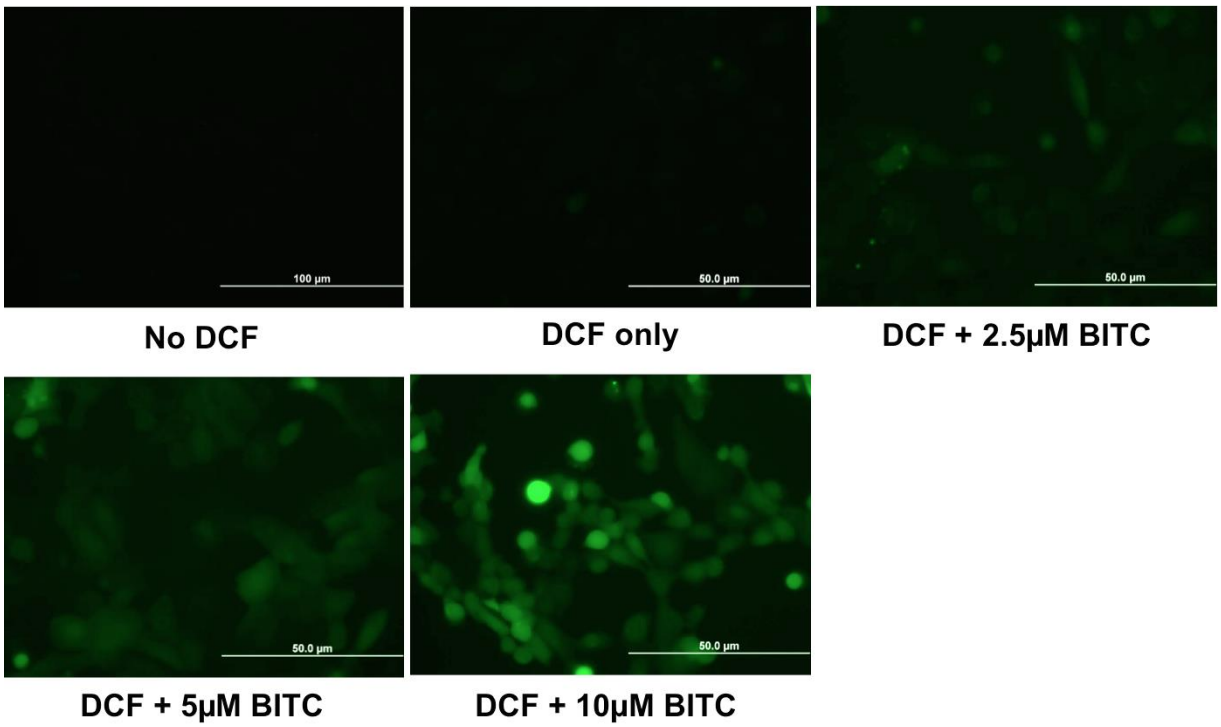

Figure 4.2 Intensity of DCF fluorescence increased in HN12 cells after BITC treatment, in a dose dependent manner.
Immunofluorescence was determined after a 1-hour treatment BITC (2.5-10µM). Representative immunofluorescence images depict DCF immunofluorescence, which correlates to ROS production. Magnification 200X.

NAC and Catalase Attenuate BITC Induced ROS

A dichlorofluorescein (DCF) assay showed that the ROS production induced by a 1-hour BITC treatment was attenuated by the addition of N-acetyl-L-cysteine (NAC) in the HN30 and HN12 cell line ($p \leq 0.05$) (**Figure 4.3).** ROS production was also significantly inhibited by catalase in the HN12 and HN30 cell line ($p \leq 0.05$), but to a lesser extent than NAC (**Figure 4.4**). Interestingly, the addition of catalase to BITC treatments kept HN30's ROS production near basal levels, but ROS production was markedly higher when catalase was added to BITC treatments in the HN12 cell line.

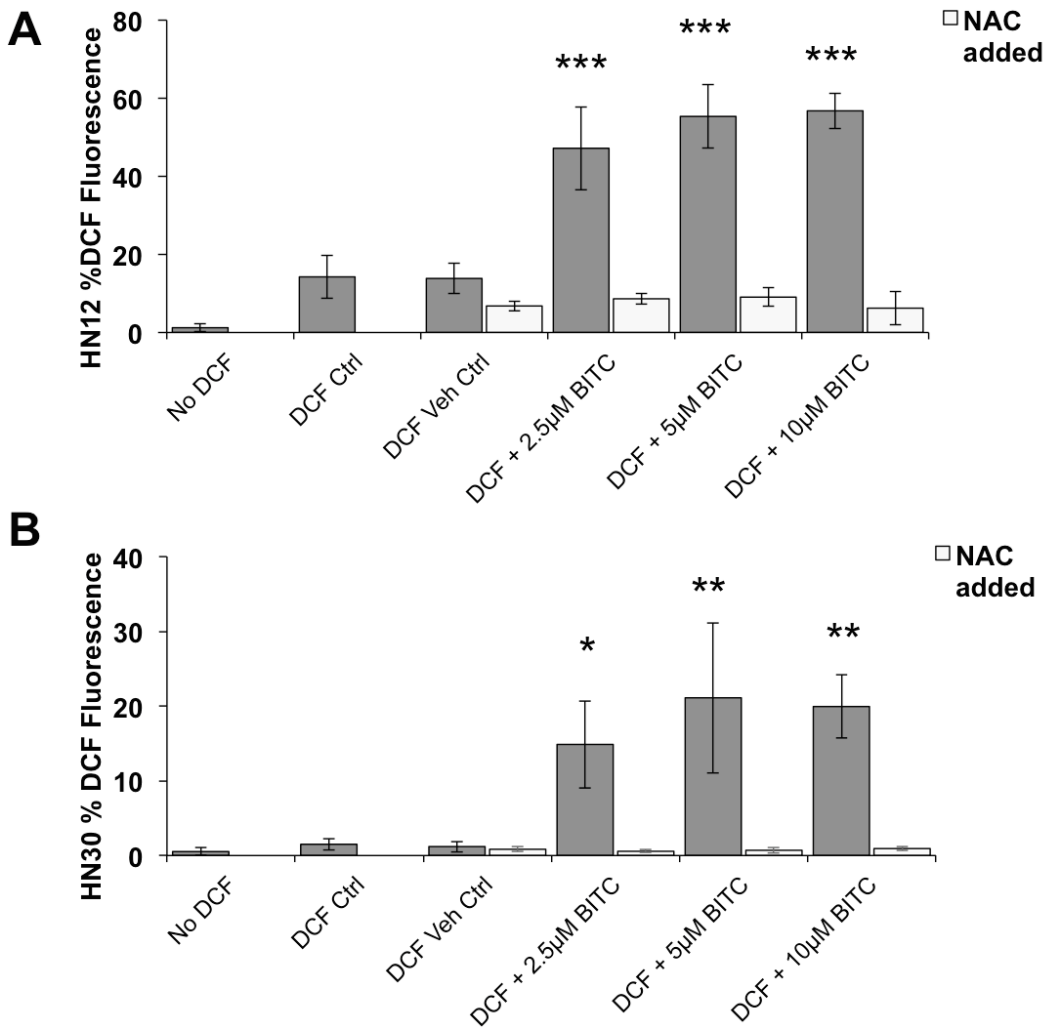

Figure 4.3 NAC inhibits BITC induced DCF fluorescence in the HN12 and HN30 cell lines.

DCF fluorescence was determined in the **A)** HN12 and **B)** HN30 cell line 24 hours after a 1-hour treatment with BITC (2.5-10μM), a 1-hour co-administration of BITC (2.5-10μM) and 5mM NAC, or a –hour treatment with 5mM NAC. DCF assay was used to determine ROS production. Dark grey bars represent cells treated with BITC only. Light grey bars represent cells co-treated with BITC and NAC. Error bars represent standard deviation. One-Way ANOVA for Multiple Comparison with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

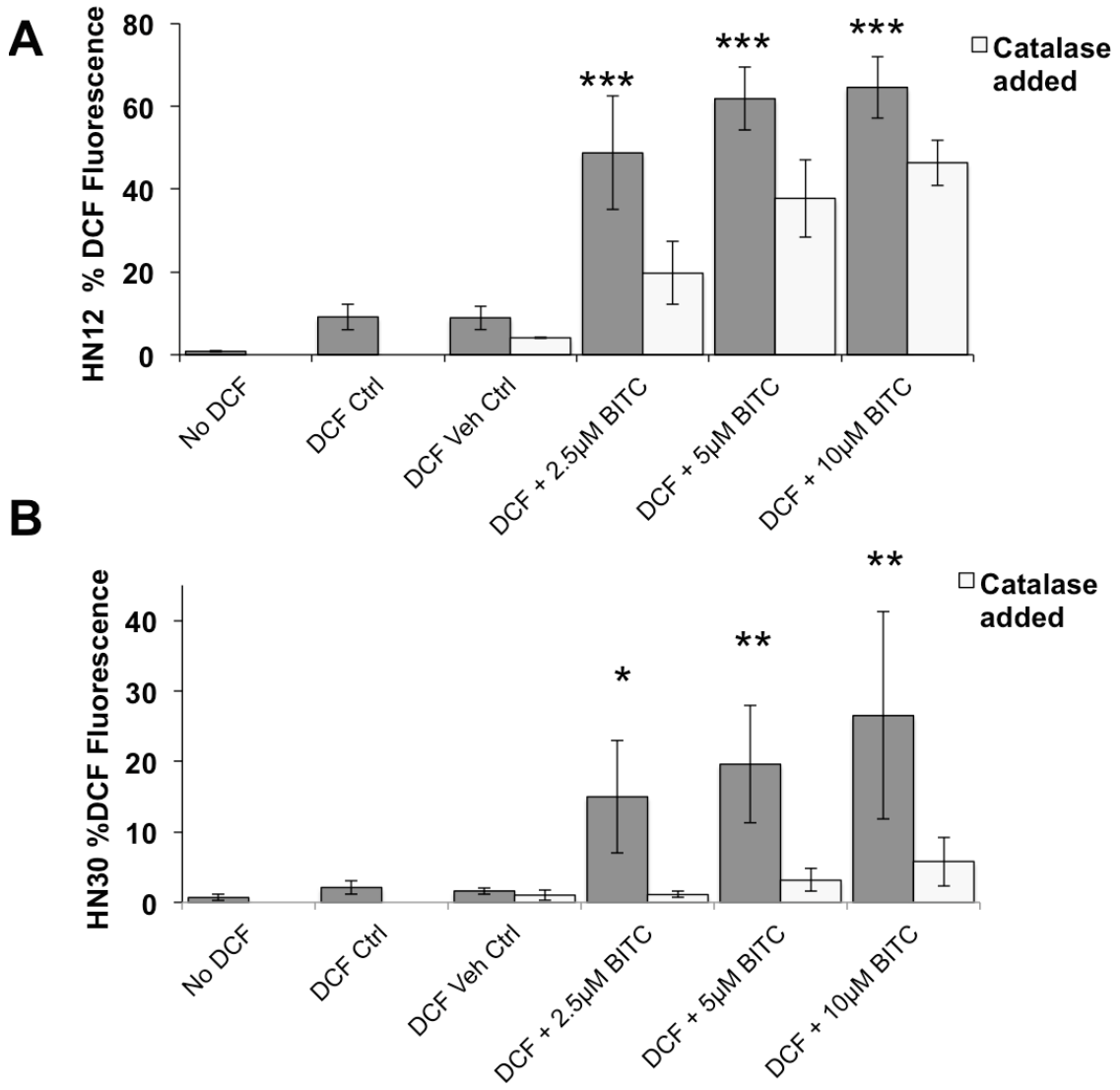

Figure 4.4 Catalase inhibits BITC induced DCF fluorescence in the HN12 and HN30 cell lines

DCF fluorescence in the **A)** HN12 and **B)** HN30 cell line after a 1-hour treatment with BITC (2.5-10μM), a 1-hour co-administration of BITC (2.5-10μM) and Catalase (2,500U/mL), or a 1-hour treatment with Catalase (2,500U/mL) alone. . DCF assay was used to determine ROS production. Dark grey bars represent cells treated with BITC alone. Light grey bars represent cells treated with BITC and Catalase. Error bars represent standard deviation. One-Way ANOVA for Multiple Comparison with Dunnet's Post-Hoc test (*$p \leq 0.05$; **$p \leq 0.001$; ***$p \leq 0.0001$).

NF-κBp50 Activity Increases after BITC Treatment

NF-κBp50 activity was significantly in the HN12 cell line after 24 hours of 10 μM BITC treatment ($p \leq 0.05$) (**Figure 4.5**). NF-κBp50 activity was non-significantly increased in the HN30 cell line after a 5 and 10 μM BITC treatment.

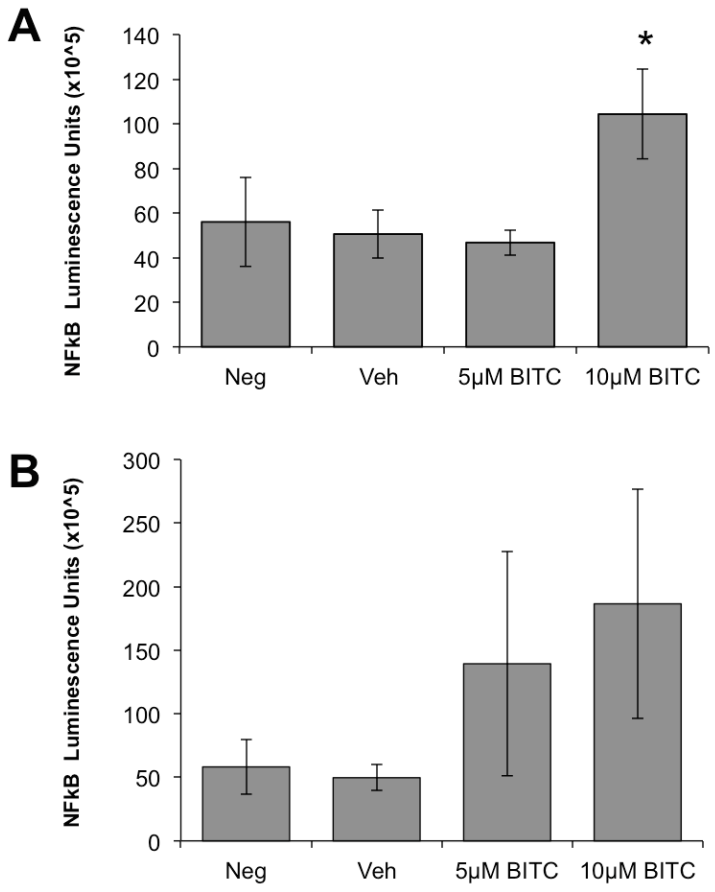

Figure 4.5 NFκBp50 activity increases in the HN12 and HN30 cell line 24hrs after BITC treatment.
NFκBp50 luminescence in the **A)** HN12 and **B)** HN30 cell line. Cells were collected 24 hours after a 1-hour treatment with BITC (5-10μM). Vehicle control was DMSO. NFκBp50 luminescence was normalized to protein concentration. Error bars represent standard deviation. Ctrl: negative control. Veh: vehicle control. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test (*$p \leq 0.05$).

The Addition of either NAC or Catalase Inhibits the Cytotoxic Effects of BITC

MTT assay results indicated that a 1-hour treatment of 5μM-10μM BITC, in DMEM with 0.5% FBS, 1% P/S significantly decreased the cell viability of the HN12 and HN30 cell lines after 24 and 48 hours ($p \leq 0.05$) (**Figure 4.6 A and B**). However, a 1-hour treatment of 1.25-2.5μM BITC in DMEM with 0.5% FBS, 1% P/S did not significantly affect the cell viability of these HNSCC cell lines after 24 hours and 48 hours (**Figure 4.6 A and B**).

Co-treatment with 5μM BITC and NAC or catalase prevented BITC from significantly reducing HN12 and HN30 cell viability after 24 and 48 hours ($p \leq 0.05$) (**Figure 4.6 and 4.7)**. Importantly, our previous work demonstrated that treatment of the normal keratinocyte cell line (HAK) with 2.5-10μM BITC did not decrease viability [44,115]. This indicates that BITC is selectively cytotoxic against HNSCC cancer cells, and that NAC and catalase can prevent the toxicity of BITC in HNSCC cells.

NAC Pre-treatment Followed by a BITC Treatment Decreased Cell Viability More Than Significantly When Compared BITC Treatment Alone

MTT assay results indicate that a 1-hour pre-treatment with NAC followed by a 2.5 or 5μM BITC treatment significantly increased the cytotoxic effects of BITC in HN12 and HN30 cells ($p \leq 0.05$) (**Figure 4.8 A and B**). Cell viability was determined at 24 hours post-treatment.

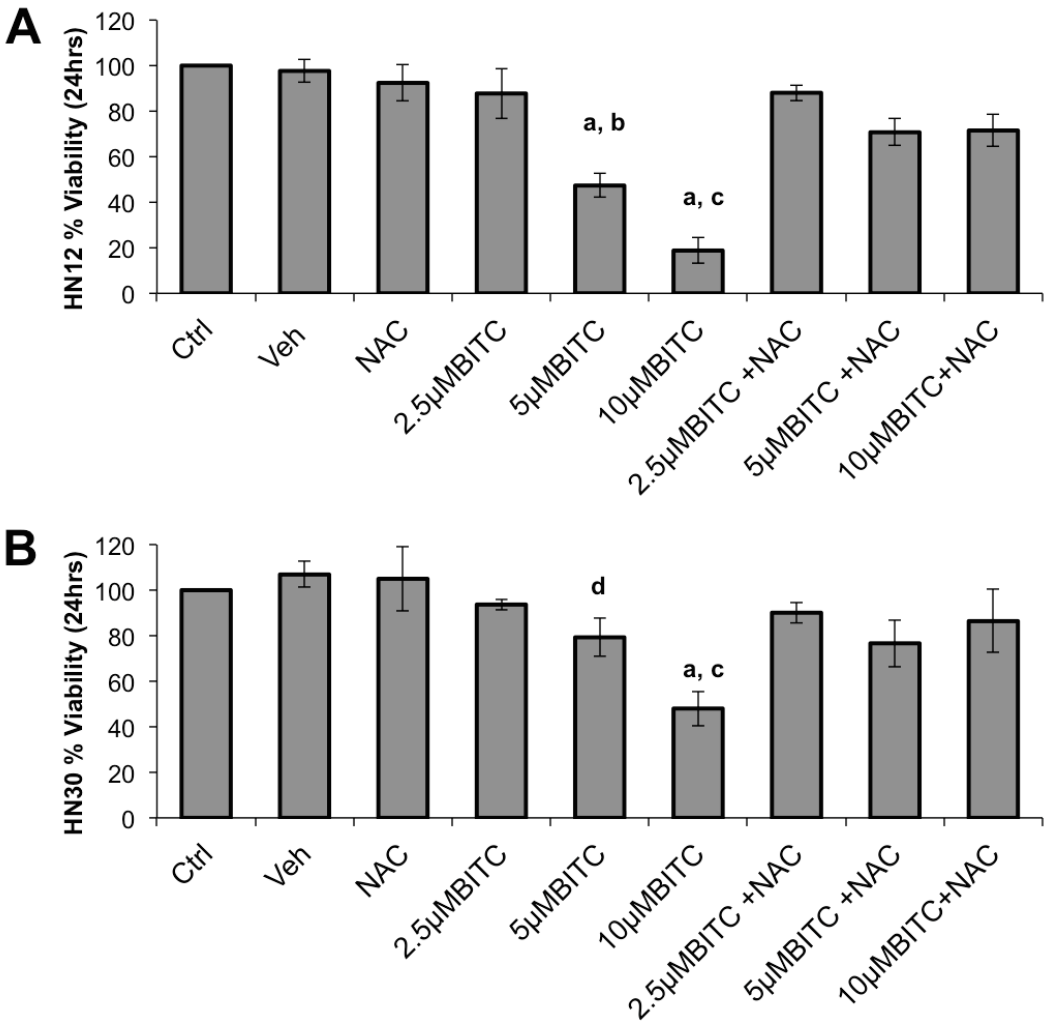

Figure 4.6 NAC co-administered with BITC prevents the effect of BITC treatments on HN12 and HN30 cell viability.
MTT assay was used to determine changes in cell viability of **A)** HN12 or **B)** HN30 cell lines 24 hours after treatment. Cells were treated with BITC (2.5-10μM), NAC+BITC (2.5-10μM), NAC, or appropriate vehicle control for 1-hour. Percent viability was determined comparing absorbance of treatment groups to their respective vehicle control. Ctrl: negative control. Veh: vehicle control. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test. **a**= significantly different from Veh Ctrl ($p \leq 0.0001$**)**; **b**=significantly different from 2.5μM BITC+NAC ($p \leq 0.0001$); **c**=significantly different from 5μM BITC+NAC ($p \leq 0.0001$); **d=s**ignificantly different from Veh Ctrl ($p \leq 0.05$).

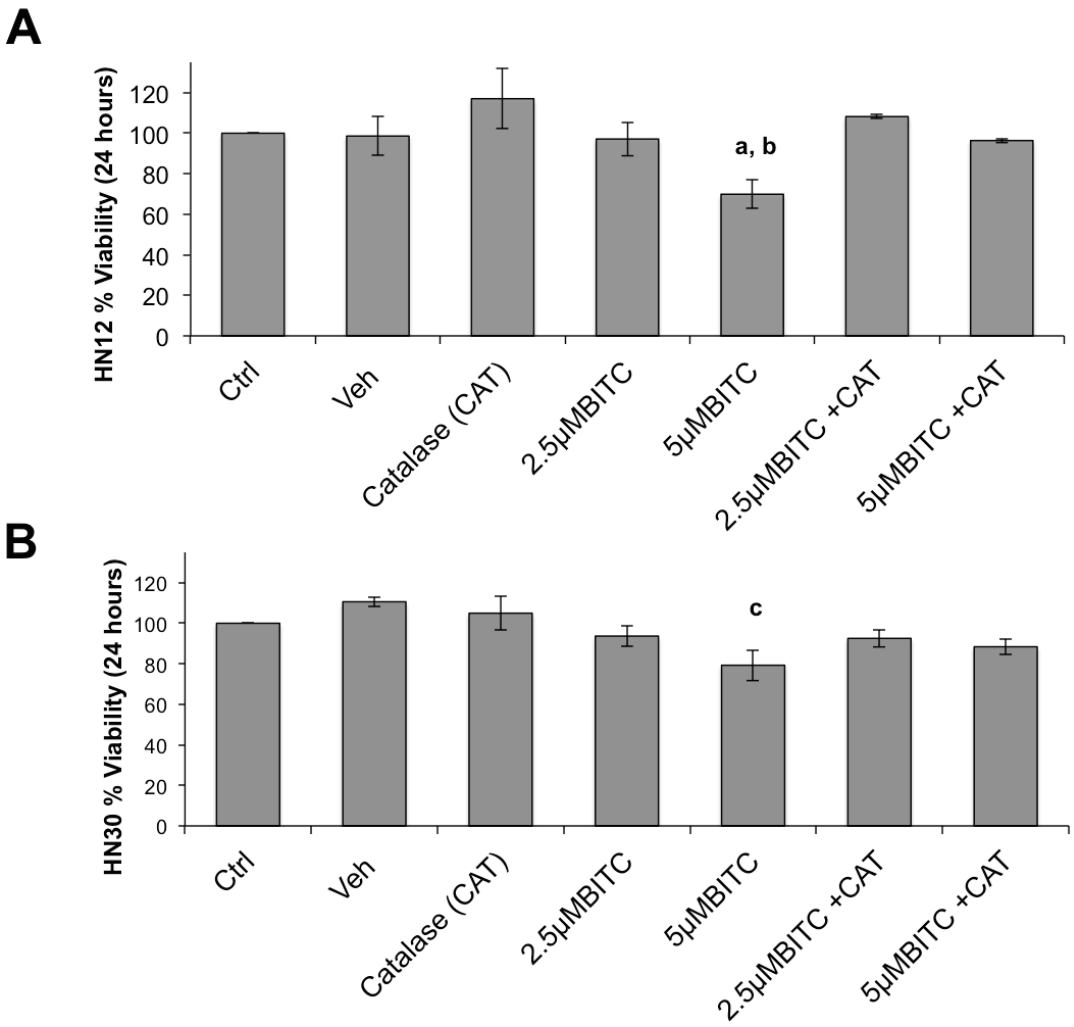

Figure 4.7 Catalase co-administered with BITC inhibits BITC treatment from reducing HN12 cell viability.

In the HN30 cell line, co-administered BITC and Catalase does not significantly alter cell viability, compared to BITC treatments alone. Cells were treated with BITC (2.5-10µM) for 1-hour, a Catalase (CAT) and BITC (2.5-10µM) co-treatment for 1-hour, Catalase alone for 1-hour, or appropriate vehicle control (VEH). MTT assay was used to determine changes in **A)** HN12 and **B)** HN30 cell viability 24 hours after treatment. Percent viability was determined comparing absorbance of treatment groups to their respective vehicle control. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test. **a**=significantly different from Veh Ctrl ($p \leq .0001$**); b**=significantly different from 5µM BITC+ Catalase ($p \leq 0.05$); **c**=significantly different from Veh Ctrl ($p \leq 0.05$)

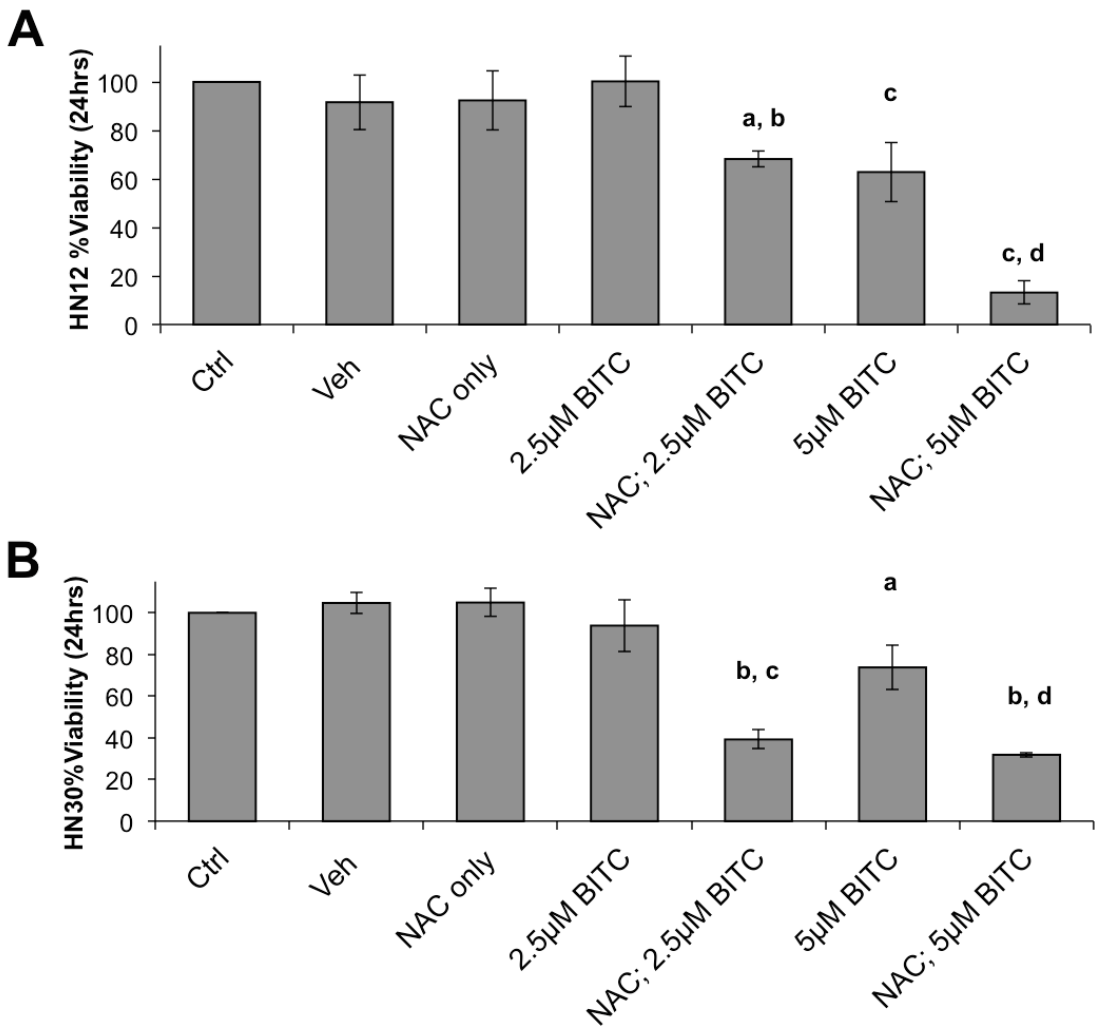

Figure 4.8 NAC pre-treatment followed by BITC treatment decreases HN12 and HN30 cell viability more significantly than BITC treatment alone.
Cells were treated with BITC (2.5-10μM) for 1-hour, a 1-hour pretreatment of NAC followed by a 1-hour treatment with BITC (2.5-10μM), NAC alone for 1-hour, or appropriate vehicle control. MTT assay was used to determine changes in **A)** HN12 and **B)** HN30 cell viability 24 hours after treatment. Percent viability was determined comparing absorbance of treatment groups to their respective vehicle control. Ctrl: negative control. Veh: vehicle control. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test. **a**=significantly different from Veh ($p \leq 0.001$**)**; **b=**significantly different from 2.5μM BITC ($p \leq 0.0001$); **c**=significantly different from Veh ($p \leq 0.0001$); **d**=significantly different from 5μM BITC *(*$p \leq 0.0001$).

NAC and Catalase Prevent BITC from Inhibiting Migration of HNSCC Cells

A wound-healing assay indicated that BITC inhibited the migration of the highly metastatic HN12 cells in a dose dependent manner, after 24 hours ($p \leq 0.05$). However, NAC (**Figure 4.9A and B**) and catalase (**Figure 4.10 A and B**) inhibited the ability of BITC to prevent wound healing in the HN12 cell line. Under the conditions used for this assay catalase treatment alone appeared to increase the wound healing, although this was not statistically significant.

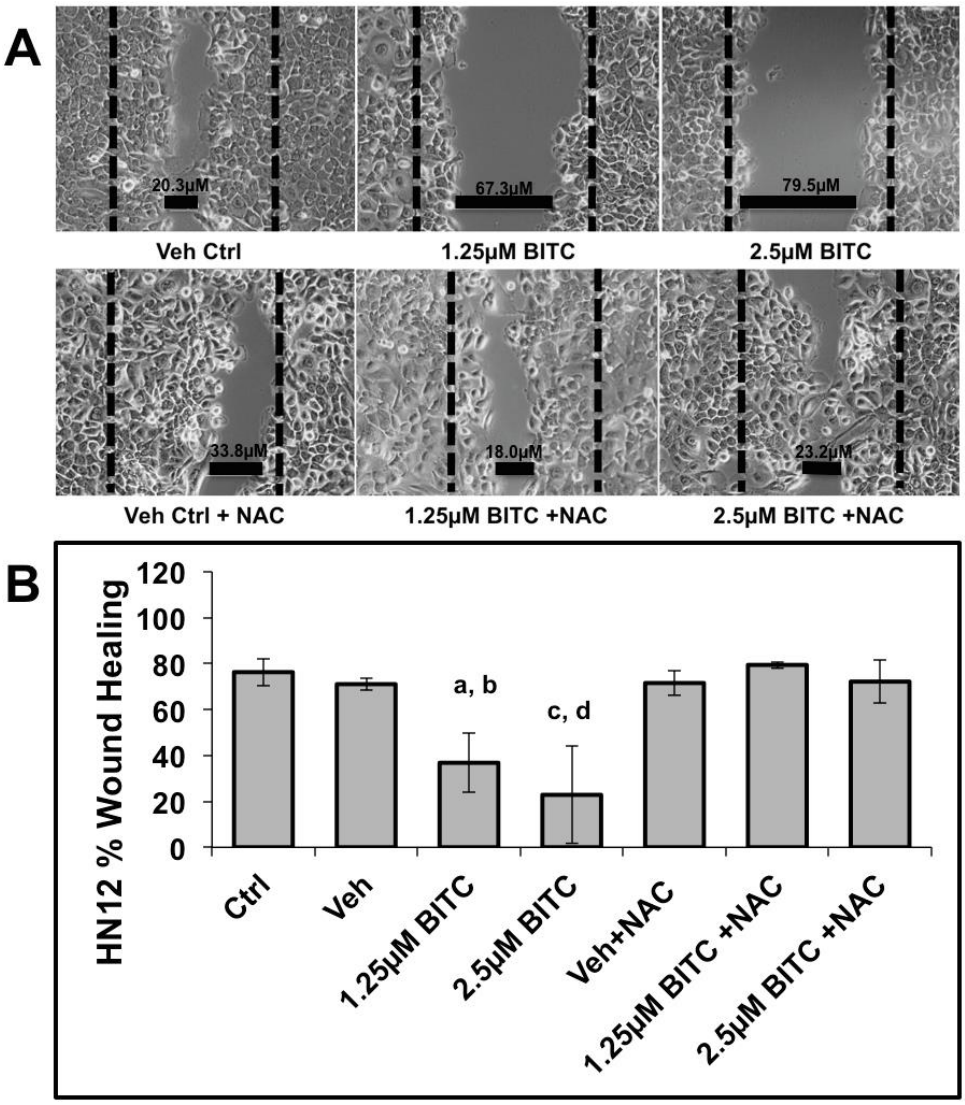

Figure 4.9 NAC co-administered with BITC prevents the effect of BITC treatments on wound healing in the HN12 cell line.
(A) Inverted light microscope images of HN12 cells 24 hours after cells were treated with BITC (2.5-10µM) for 1-hour, a NAC and BITC (2.5-10µM) co-treatment for 1-hour, NAC alone for 1-hour, or appropriate vehicle control. Dashed lines represent scratch size before treatment. Magnification 100X. (**B**) Bar diagram represents the percent wound healing of the HN12 cell line 24 hours after treatment. Percent wound healing was calculated using wound size measurements. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test. **a**= significantly different from Veh ($p \leq 0.05$); **b**= significantly different from 1.25µM BITC+NAC ($p \leq 0.05$)**; c**= significantly different from Veh ($p \leq 0.0001$); **b**= significantly different from 2.5µM BITC+NAC ($p \leq 0.0001$). Ctrl: negative control. Veh: vehicle control.

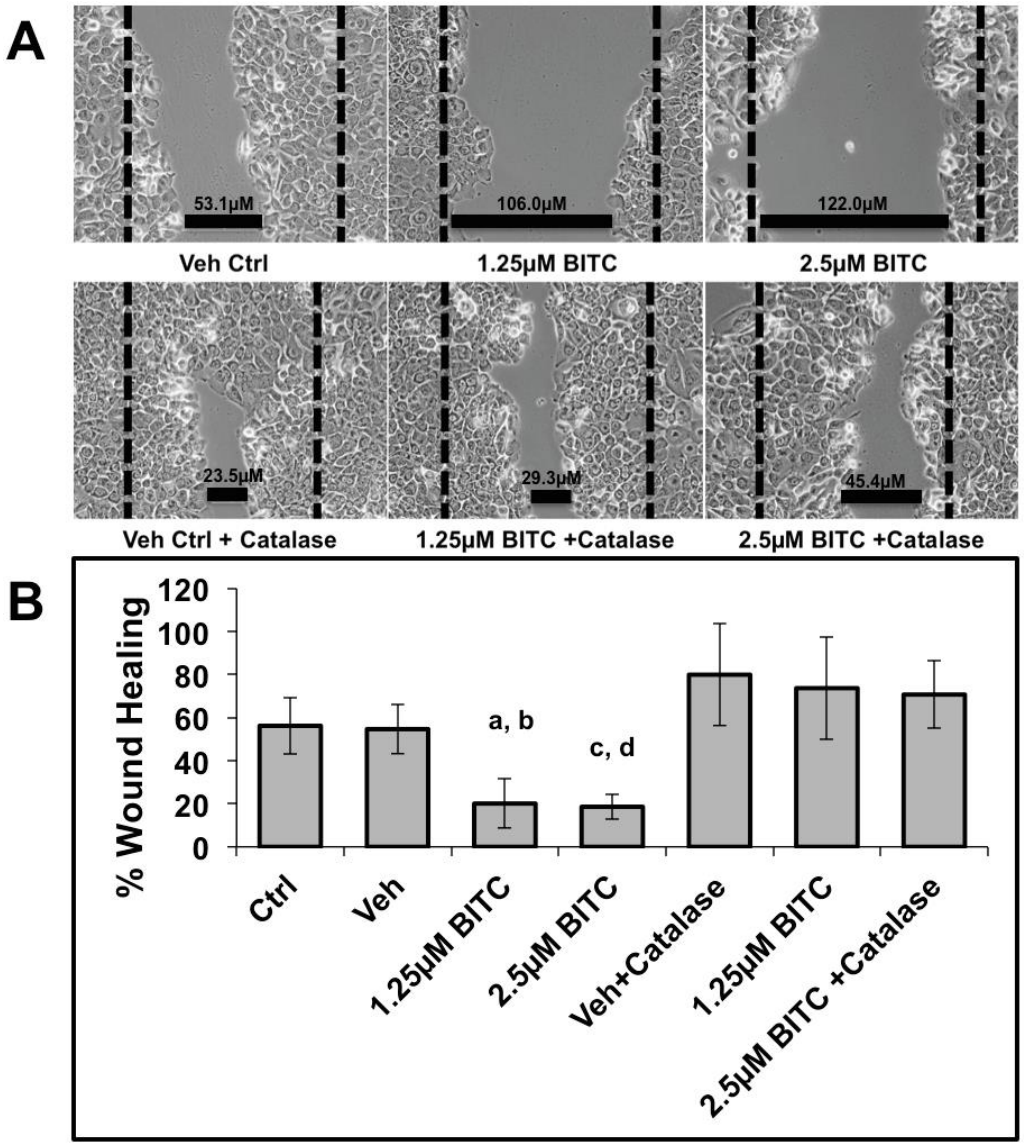

Figure 4.10 Catalase co-administered with BITC prevents the effect of BITC treatments on wound healing in the HN12 cell line.
(A) Inverted light microscope images of HN12 cells 24 hours after cells were treated with BITC (2.5-10µM) for 1-hour, a Catalase and BITC (2.5-10µM) co-treatment for 1-hour, Catalase alone for 1-hour, or appropriate vehicle control. Dashed lines represent scratch size before treatment. Magnification 100X. **(B)** Bar diagram represents the percent wound healing of the HN12 cell line 24 hours after treatment. Percent wound healing was calculated using wound size measurements. Error bars represent standard deviation. One-way ANOVA for multiple comparisons with Dunnet's Post-Hoc test. **a**=significantly different from Veh ($p \leq 0.001$**);** **b**=significantly different from 1.25µM BITC+Catalse ($p \leq 0.0001$)**;** **c**= significantly different from Veh ($p \leq 0.0001$); **d**=significantly different from 2.5µM BITC+Catalase ($p \leq 0.0001$). Ctrl: negative control. Veh: vehicle control.

NAC Prevents BITC Induced Chemo-enhancement

One of the major obstacles in the therapeutic use of platinum analogues is intrinsic or acquired resistance [100-103]. Previous work from our lab indicated that BITC treatment enhanced the effects of cisplatin (CDDP) in CDDP resistant HNSCC cell lines [115]. Our recent results suggest that when NAC is added to 1-hour pretreatment of BITC the effects typically induced by a pretreatment of BITC followed by CDDP are abrogated at the 24 and 48-hour time points in the HN12 and HN30 cell lines ($p \leq 0.05$) (**Table 4.1**).

Table 4.1 Percent viability of HN12 and HN30 cell lines 24 and 48 hours after treatment*.

Treatment Group	HN12 Cell Line		HN30 Cell Line	
	24hr	48hr	24hr	48hr
NAC	92.49 ± 8.0 [c;d;e]	94.04 ± 4.1 [c;d;e]	104.96 ± 8.3 [c;d;e]	82.54 ± 12.3 [c;d;e]
5μM BITC	47.33 ± 5.2 [a;b;d]	30.24 ± 1.8 [a;b;d;e]	79.28 ± 7.6[a;b;d]	54.44 ± 6.23[a;b;d]
5μM BITC +NAC	70.72 ± 5.9 [a;b;c;d;e]	87.85 ± 1.71 [a;c;e]	76.66 ± 4.5 [a;b;c;d;e]	89.57 ± 10.8 [b;c;d;e]
5μM BITC; 10μM CDDP	30.68 ± 3.6 [a;b;c;d]	11.64 ± 1.49 [a;b;e]	41.54 ± 10.32 [a;b;c;d]	18.95 ± 7.6 [a;b;c;d]
5μM BITC +NAC; 10μM CDDP	101.32 ± 9.2 [b;c]	64.71 ± 8.2 [a;c;e]	83.05 ± 13.8 [c;e]	48.08 ± 17.7 [a;b;e]
10μM CDDP	70.37 ± 13.2[b;c]	60.58 ± 10.1 [a;c;e]	62.53 ± 5.5 [a;b;c;d]	50.33 ± 6.06 [a;b;e]
NAC; 10μM CDDP	86.34 ± 12.3 [b;e]	64.35 ± 13.2 [a;b;e]	81.47 ± 12.3 [b;e]	52.00 ± 9.78 [a;b;e]

*****Percent viability ± standard deviation was calculated comparing MTT absorbance of treatment groups to their respective vehicle control. Cells were treated with BITC alone for 1-hour, NAC alone for 1-hour, Cisplatin (CDDP) alone for 24-hours, co-administration of BITC and NAC for 1-hour, NAC for 1- hour followed by CDDP for 24-hours, a 1-hour pretreatment of BITC followed by a treatment of CDDP for 24 hours; or a 1-hour pretreatment of BITC and NAC followed by a treatment of CDDP (10μM) for 24 hours. **a**=significant difference from respective Vehicle Control; **b**=significant difference from Vehicle Control + NAC; **c**=significant difference from 5μM BITC; **d**=significant difference from 10μM CDDP; **e**=significant difference from a 5μM BITC pretreatment followed by a 10μM CDDP

DISCUSSION

Previous studies from our lab indicated that BITC has a dual function of targeting both cell viability and cell migration [44,116]. BITC, like other ITCs, targets a variety of molecular pathways making it difficult to pinpoint how BITC elicits its therapeutic response in HNSCC cells [2,116]. However, the results of the present study demonstrate that BITC induced ROS regulates both HNSCC cell viability and migration. By attenuating BITC induced ROS with NAC and catalase we were able to inhibit BITC's ability to prevent cell migration and add to the effects of cisplatin.

NAC and catalase are both antioxidants that scavenge free radicals. NAC is a GSH precursor and thereby increases cellular GSH [23,117,118]. Cellular GSH plays a central role in maintaining redox homeostasis, and reduced GSH act as a direct scavenger of ROS by reacting with singlet oxygen, hydroxyl radicals, and superoxide radicals [23]. We used NAC to investigate a mechanism of action of BITC in HNSCC cells. NAC in combination with ITCs has been used to investigate ITC's mechanism of action in other cell lines, and recently Gong *et al.* (2009) used NAC in combination with ITCs to support that ROS alters STAT3 activation in prostate cancer cells [14].

In our study BITC treatments significantly increased ROS production in HNSCC cell lines. An increase in NF-κBp50 activity in HNSCC cells supports that the increased ROS has downstream effects. However, an increase in NF-κBp50 is novel and an inhibition of NF-κBp50 is observed in other cancer cell lines.

BITC initiated ROS production was significantly decreased when NAC and BITC were co-administered. Additionally, NAC prevented BITC induced cytotoxicity. However, our data

supports that the timing of NAC administration is critical to its ability to attenuate the effects of BITC. In our experiments, when NAC was administered an hour before BITC treatment its effects were markedly different. NAC pretreatment enhanced BITC induced cytotoxicity. High levels of cellular GSH support isothiocyanate uptake, and this could explain why pretreatment of NAC increases BITC induced cytotoxicity [11,105,119,120]. NAC also alters redox status in cells and we speculate that the pretreatment with NAC could increase the effects BITC induced ROS, whereas when NAC and BITC were co-administered, NAC inhibited BITC induced ROS [11,35,110]. This highlights the challenge of understanding the link between ROS, BITC, and cellular function.

More investigation on the relationship between BITC and NAC will be needed, but the relationship between BITC and NAC co-administration should not be overlooked. Regardless of why NAC inhibited ROS production, it is still evident that NAC prevented the therapeutic effects of BITC when co-administered. This knowledge is still critical for future clinical trials. Our results stress the importance of a monitoring a patient's antioxidant consumption, because our data supports that administering BITC with NAC would have not been beneficial for a patient. Our data is also interesting because other research groups have found that NAC co-administration enhances the effects of certain anti-cancer agents, such as retinoic acid [121,122].

Catalase is a scavenger of H_2O_2 and we used this reagent to further support the NAC studies. Catalase significantly prevented BITC induced ROS production in the HNSCC cell lines. The prevention of ROS production was not to the same extent as NAC, probably because catalase is more specific in its ROS scavenging abilities. Catalase did, however, highlight the potential of H_2O_2 induction in HNSCC cell lines. Furthermore, catalase alone appeared to increase HNSCC cell migration, which is interesting because several reports suggest that

increasing ROS production is usually associated with increased cellular migration [112,113]. Our results are supported by Luanpitpong *et al.* (2010) and because their study also showed that catalase stimulated cell migration while H_2O_2 inhibited cellular migration [114]. Inokuma *et al.* (2012) also suggest that increased ROS is pertinent to preventing lymph node metastasis in colorectal cancer [123]. Furthermore, Das *et al.* (2012) report that ROS generation inhibits EMT and promotes growth arrest in prostate cancer cells [124]. Taken together, our results combined with others indicate that investigating ROS as a whole may lead to confounding results. Our results support the notion that specific types of ROS combined with the molecular pathways that are altered in certain cancer cells can have nontraditional results.

In addition to preventing cellular migration we also showed that NAC and catalase prevented BITC induced cytotoxicity in the HNSCC cells. NAC also prevented BITC's ability to enhance the effects of the cisplatin in reducing cell viability. The platinum compound, cisplatin, is regarded as one of the standard-of-care treatments for HNSCC, and is typically used in combination radiation or other agents [78,125,126]. Initial or acquired resistance to platinum compounds greatly hinder outcome for HNSCC patients and high levels of drug detoxifying agents are linked to cisplatin resistance [126]. Two such drug-detoxifying agents linked to cisplatin resistance are high levels of GSH and catalase.

However, one way cisplatin induces cell death is by increasing ROS. This evidence supports the mechanism that lower cellular ROS lead to cisplatin resistance. Therefore, using NAC to increase cellular GSH and catalase to scavenge H_2O_2 we could be preventing the mechanism through which BITC enhances cisplatin toxicity. Our results showing that by pretreating the HNSCC cells with BITC the ROS were increased and could explain why we

observed increased cell death in platinum-resistant HNSCC cells pretreated with BITC followed by cisplatin.

In conclusion, the present study sets the framework for future investigation into the mechanism through which BITC inhibits migration and invasion, as well as induces chemoensensitivity. Our study suggests that ROS generation is one important mechanism by which BITC elicits its response. Future studies, will work to continue to unravel the role of ROS in HNSCC by including experiments to evaluate the role of antioxidants, like vitamin E that affects the lipid bilayer, to further improve our understanding of mechanisms involved in chemoresistance or chemosensitivity of HNSCC cells. Additionally, future *in vivo* studies will shed the greatest light on the potential and feasibility of BITC as an adjuvant to anti-cancer agent.

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Chapter 5 **: Conclusion and Future Direction**

In Chapter 1, we reviewed current literature on ITCs and highlighted the potential of ITC treatments as adjuvant cancer therapy. We chose to specifically focus on ITC as a therapy for HNSCC, because, as discussed in Chapter 2 the quality of life in response to current therapy for advanced HNSCC is poor. We focused specifically on BITC as an additive to current chemotherapy because this ITC elicited the strongest anti-tumor response in HNSCC cells. In Chapter 3, we demonstrated that after 24 and 48 hours BITC treatment significantly reduced the viability of multiple HNSCC cell lines tested (HN12, HN8, and HN30), but not a normal keratinocyte line (HAK). Treatment with BITC also decreased migration and invasion of the HN12 cell line and inhibited expression and altered localization of the EMT marker, vimentin. We also reported that a pretreatment of BITC followed by a treatment with cisplatin decreased HN12, HN30, and HN8 cell viability and proliferation greater than when compared to either treatment alone. Pretreatment with BITC followed by a cisplatin treatment also increased apoptosis more than either treatment alone. Additionally, BITC treatment decreased expression of the anti-apoptotic protein, bcl-2.

Current therapies can be further improved if we understand the mechanism through which our treatments elicit and anti-tumor response. In Chapter 4, we discussed how treatments with BITC significantly increased ROS production in HNSCC cell lines. NAC and catalase attenuated this increase in ROS production. ROS is a complex topic and presents an interesting paradox in cancer. Literature supports the hypothesis that ROS can be carcinogenic or suppress cancer progression. Our particular study indicates that in this model ROS production elicits and anti-cancer response, because at low concentrations BITC induced ROS production and limited

HNSCC cell migration however, when using higher concentrations of BITC, ROS decreased cell viability.

Literature reveals that ITCs, including BITC, target many molecular pathways and our present body of work suggest that increased in ROS is one mechanism through which BITC treatments targets HNSCC cells. As previously mentioned, ROS is a complex topic in relation to cancer therapy. Our investigation only begins to unravel the role of ROS in HNSCC. More work will be needed to fully understand the role of BITC induced ROS. Such studies would incorporate other anti-oxidant products into BITC treatments. Vitamin E is one example of an anti-oxidant that would increase our understanding of mechanism involved in BITC induced ROS in HNSCC cells, because it is a lipid soluble anti-oxidant that prevents lipid membrane peroxidation. Signaling from the lipid membrane can alter many cellular functions. Additional studies investigating the effect of BITC on GSH could increase our knowledge of BITC's mechanism of action. Preliminary data also suggest that studying the effect of BITC on multi-drug transporters could highlight additional targets of BITC.

In addition the experiments suggested above, we propose several other future studies to further increase our understanding of BITC's potential as an adjuvant therapy. Our preset investigation only studied one type of HNSCC therapy in conjunction with BITC. The potential of BITC to add to or synergize other HNSCC therapies is high. Developing an HNSCC animal model to study BITC as an adjuvant therapy will increase our understanding of BITC in an *in vivo* setting. Future work should investigate BITC in combination with other chemotherapies as well as radiation therapy.

A shortcoming of our present studies is the lack of *in vivo* data, however, studies by other groups suggest that *in vitro* results correlate with *in vivo* observations.

In conclusion, our present body of work supports that BITC is a promising adjuvant chemotherapy for HNSCC. The studies on BITC presented in this thesis suggest that this therapeutic option is worth exploring on a larger scale. Progression of our current study from bench to bedside will ultimately determine if inclusion of BITC as adjuvant therapy option for HNSCC will improve patient outcome and quality of life.

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APPENDIX

Office of Research Integrity IRB Approval

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

January 22, 2014

Mary Allison Wolf
MUSOM/MTGRI
Room 276
1600 Medical Center Drive
Huntington, West Virginia 25701

Dear Ms. Wolf:

This letter is in response to the submitted thesis abstract for the *in vitro* study to evaluate the use of benzyl isothiocyanate (BITC) as a treatment for head and neck squamous cell carcinoma (HNSCC). After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

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

Sincerely,

Bruce F. Day, ThD, CIP
Director

Curriculum Vitae

Mary Allison (Teter) Wolf

teter6@marshall.edu

EDUCATION

Marshall University · Huntington, WV (2009-present)

- Biomedical Sciences Graduate Program; GPA: 3.72
- Ph.D. Candidate –Cancer Biology

WV Wesleyan College · Buckhannon, WV (2005-2009)

- Bachelors of Science, Department of Biological Sciences
- Graduated Magnum Cum Laude; GPA: 3.857

HONORS & AWARDS

2014	Marshall University School of Medicine Research Day Best Oral Presentation (Basic Science)
2013	Marshall University School of Medicine Research Day Best Oral Presentation (Basic Science)
2013	Selected to give American Society for Nutrition (ASN) oral presentation (EB meeting)
2012	Best Overall Performance as a Graduate Student, International Travel Award
2012	ASN Diet and Cancer Mini-Symposium Poster Presentation Award (3rd)
2012	Dr. Frederick J. Lotspeich Scholarship in Biomedical Sciences
2012	Cell Differentiation and Development Center Symposium Poster Presentation Award (First Place)
2012	Anagene B. Heiner Memorial Award for Best Basic Science Poster Presentation
2009	Inducted into Phi Kappa Phi, Mortar Board, Omicron Delta Kappa Honoraria's
2009	Most Outstanding Oral Presentation at Mid-Atlantic Research Conference
2009	WV Wesleyan Most Outstanding Student Research Award
2007-2009	Beta Beta Beta Biology Honorary, Vice President

SERVICE

2013	Spearheaded the planning for the first Teen Science Café in Huntington, WV. Partnered with the American Society for Biochemistry and Molecular Biology to prepare a meeting with local high school students. Goal of the Science Café is to raise awareness and interest about biomedical research.
2012	Organized the Jared Box Project donation drive for the BMS Graduate Student Organization (GSO). Delivered toys to Cabell Huntington Hospital and St. Mary's Medical Center
2012	Planned the first Appalachian Regional Cell Conference (ARCC). ARCC was a student driven research conference held in October in Charleston, WV.
2012	Student mentor for incoming Ph.D. students

2012	Planned a GSO fundraiser to send medical supplies with a colleague to Uganda
2011	Organized the first GSO scholarship to be given annually to BMS Ph.D. and Masters Students; on selection committee (2011, 2012)
2011	Bake Sale/Donation Drive organizer for The Jared Box. We delivered toys to children at Cabell Huntington Hospital before Christmas.
2011	Organized a GSO Canned Food Drive for a local shelter
2009	Breast Cancer Awareness Bounce-a-Thon and candlelight vigil organizer; raised over $1,000 for the Zeta Tau Alpha Breast Cancer Foundation

COMMITTEES

2009-present	Biomedical Sciences Graduate Student Organization; President (‘12-present); Vice President (‘11-‘12)
2012	Biomedical Sciences Website Committee (re-designed Marshall’s BMS website)
2011-2012	Biomedical Sciences Graduate Counsel Student Representative

GRANTS

2013-2014	WV-NASA SGC **TITLE:** Investigation of benzyl isothiocyanate’s regulation of metastatic processes in HNSCC cell lines.
2013	American Society for Cell Biology Meeting Award to organize a student driven research conference, Co-PI
2012-2013	WV-NASA SGC **TITLE:** Exploring BITC’s Ability to Enhance Chemosensitivity and Reduce the Metastatic Potential of Head & Neck Cancer Cells
2012	American Society for Cell Biology Meeting Award to organize a student driven research conference, Co-PI
2011-2012	WV-NASA SGC **TITLE:** Chemosensitization of Non-Small Lung Cancer Stem Cells with Isothiocyanates
2008-2009	Summer Undergraduate Research (SURE) Grant, **TITLE:** Antibiotic and Sodium Hypochlorite Susceptibility in *Pseudomonas aeruginosa*.

Skills

Cell Culture	Animal Models	Virus Purification
PCR	Western blots	Molecular Cloning
Flow Cytometry	Fotodyne Imaging Software	Microscopy

PROFESSIONAL AFFILIATION

2012-present	American Society for Cell Biology
2012-present	American Society for Nutrition
2008-2010	American Society for Microbiology

TEACHING EXPERIENCE

Fall 2013	Biomedical Sciences Teaching Practicum, taught 4 hours of Biochemistry and Cell Biology (BMS 600)

Spring 2013	Biomedical Sciences Teaching Practicum, taught 4 hours of Cancer Biology (BMS 651)
Fall 2012	Biomedical Sciences Teaching Practicum, taught 3 hours of Biochemistry and Cell Biology (BMS 600)
2007-2009	Level One Biology Tutor at WV Wesleyan College, Tutored Principles of Biology, General Biology, Human Anatomy, and Genetics
Sp' 2009	General Biology and Molecular Cell Biology Lab Teaching Assistant
Fall 2009	Principles of Biology Lab Teaching Assistant
Sp' 2008	Organic I Lab Teaching Assistant

PRESENTATIONS

2011	Guest Lecturer at WV Wesleyan College, Alderson Broaddus College; **Topic:** Cancer Stem Cells and Isothiocyanates
2011	Guest Lecturer at Davis and Elkins College; **Topic**: What to expect from a BMS Graduate School

STUDENTS MENTORED

2010-2013	Oksana Bailiff, Marshall University Medical Sciences Masters Student (2.5 years)
2010	Allison Polumbo, Marshall University School of Medicine Student (2 months)
2010	Lauren Strait, University of Louisville Undergraduate Student (3 months)

PEER-REVIEWED PUBLICATIONS

Wolf, M.A. and P.P. Claudio (**2014)**. Benzyl isothiocyanate reduces the metastatic potential and enhances chemosensitivity of head and neck squamous cell carcinoma cells. Nutrition and Cancer: 1-10

Damron, F.H., J. Napper, **M.A. Teter**, and H.D. Yu. (**2009)**. Lipotoxin F of *Pseudomonas aeruginosa* is an AlgU-dependent and alginate-independent outer membrane protein involved in resistance to oxidative stress and adhesion to A549 human lung epithelia. Microbiology 55: 1028-1038.

OTHER PUBLICATIONS

Wolf, M.A. and P.P. Claudio (**2013**). Isothiocyanates target carcinogenesis during tumor initiation, promotion, and progression/Article. In P.P. Claudio & R.M. Niles (Eds.), *Nutrition and Cancer: From Epidemiology to Biology* (p79-86). Oak Park, IL: Bentham Science Publishers Ltd

SELECTED ABSTRACTS and POSTERS ('07-present)

1. **Wolf, M.A.** and P.P. Claudio. (**March 2014**). Benzyl isothiocyanate sensitizes cells to cisplatin, and inhibits HNSCC cell migration and invasion. JCESOM Research Day, Huntington, WV. **(selected to give oral presentation)**
2. **Wolf, M.A.** and P.P. Claudio. (**May 2013**). Benzyl isothiocyanate enhances chemotherapy and reduces migration and invasion of head and neck squamous cell carcinoma cells. 4th World Congress of the International Academy of Oral Oncology, Rhodes Island, Greece.
3. **Wolf, M.A.** and P.P. Claudio. (**March 2013**). Investigating benzyl isothiocyanate as a treatment option for head and neck squamous cell carcinoma. Experimental Biology 2013, Boston, MA. (**selected to give American Society for Nutrition oral presentation**)

4. **Wolf, M.A.** and P.P. Claudio. (**March 2013**). Benzyl isothiocyanate enhances chemotherapy and reduces migration and invasion of head and neck squamous cell carcinoma cells. JCESOM Research Day, Huntington, WV. **(selected to give oral presentation)**
5. **Wolf, M.A.**, A. Polumbo, W.E. Hardman, P.P. Claudio. (**2012**). Benzyl isothiocyanate targets chemoresistant and metastatic head and neck squamous carcinoma cells. Experimental Biology Conference, San Diego, CA; **ASN mini-symposium Poster Presentation Award (3rd)**
6. **Wolf, M.A.**, A. Polumbo, W.E. Hardman, P.P. Claudio. (**2012**). Benzyl isothiocyanate enhances chemosensitivity and decreases migration of head and neck squamous carcinoma cells. CDDC mini-symposium, Huntington, WV; **Poster Presentation Award (1st)**
7. **Wolf, M.A.**, A. Polumbo, W.E. Hardman, P.P. Claudio. (**2012**). Benzyl isothiocyanate enhances chemosensitivity and decreases migration of head and neck squamous carcinoma cells. CDDC mini-symposium, Lexington, KY
8. **Wolf, M.A.**, A. Polumbo, W.E. Hardman, P.P. Claudio. (**2012**). Benzyl isothiocyanate targets chemoresistant and metastatic head and neck squamous carcinoma cells. JCESOM Research Day, Huntington, WV; **Poster Presentation Award (1st)**
9. **Wolf, M.A.**, O. Bailiff, J. Valluri, and P.P. Claudio. (**2011**). Isothiocyanates decrease viability of pancreatic cancer cell lines. Sigma Xi Research Conference, Huntington, WV
10. **M. A. Teter**, F.H. Damron, M.K. Bartley, and H.D. Yu. (**2009**). Lipotoxin F mediated antibiotic susceptibility and biofilm formation in *Pseudomonas aeruginosa*. Mid-Atlantic Undergraduate Research Conference, Buckhannon, WV; **Most Outstanding Oral Presentation Award**
11. **M. A. Teter**, F.H. Damron, M.K. Bartley, and H.D. Yu. (**2008**). OprU mediated antibiotic susceptibility and biofilm formation in *Pseudomonas aeruginosa*. WV-INBRE Research Poster Symposium at WVU, Morgantown, WV
12. **Teter, M.A.**, S.L. Edenborn, H.M. Edenborn, and S.K. Owen. (**2008**). Nutritional similarities between mannitol salt negative bacteria isolated from the nasopharyngeal cavities of college athletes. The 108th General Meeting of the American Society for Microbiology, Boston, MA
13. **M. A. Teter**, A. K. Holley, M. R. Moore. (**2007**). Progestin Stimulation of Migration of T47D Human Breast Cancer Cells. STaR Symposium at WVU, Morgantown, WV; **Poster Presentation Award (Honorable Mention)**