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Effect of Aryl Hydrocarbon Receptor Modulation on HNSCC Survival

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ABSTRACT

Despite current therapies, at least 50% of patients with locally advanced head and neck squamous cell carcinomas (HNSCC) develop either locoregional or distant relapses within 2 years of treatment. Existing research regarding relapse suggested a role of the aryl hydrocarbon receptor (AHR) in the aggressive HNSCC phenotype perhaps contributing to the relapse of many chemotherapy patients. More specifically, AHR activation by AHR agonists such as dioxin or polycyclic aromatic hydrocarbons (found in tobacco smoke) has repeatedly been shown to play a role in antiapoptotic activity and cell survival. Under nutrient-deprived conditions relevant to HNSCC solid tumors, this may in turn serve a role in promoting tumor cell survival. Likewise, the application of AHR antagonists may in turn also play a role in making tumors much more susceptible to chemotherapeutic agents, thus increasing their efficacy. The purpose of the project as such is to assess the ability of HNSCC cell line HN30 cells to resist the effects of chemotherapeutic agents in the presence of AHR agonists/antagonists under tumor-relevant conditions utilizing a monolayer culture system.

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Introduction

The Aryl Hydrocarbon Receptor

The Aromatic Hydrocarbon Receptor (AHR) is a ligand-mediated transcription factor commonly known for its ability to mediate xenobiotic (drug) metabolism via inducement of detoxifying enzymes and cell immunity modulation.[1] The function of AHR as a regulator of gene expression itself have been shown to play a role in various biochemical pathways. Recent studies via DNA microarray and quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) have shown that AHR activation plays significant role in altering expression of genes involved in energy metabolism, circadian rhythm, lipid cholesterol metabolism, along with other relevant pathways.[2,6] Furthermore, AHR has also been found to play a significant homeostatic role, with functions ranging from T Helper cell differentiation to cytokine signaling and regulations of hormonal response.[3] Physiologically, the highest expression of AHR have been observed primarily in the liver, lungs, spleen, and kidney, though varied expression levels have been portrayed in virtually all except that of skeletal muscle tissue.[3] Similarly, of the cells that express AHR, epithelial cells were seen to have the highest expression relative to other cell types.[3]

Structurally, the AHR is a member of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) group, commonly known as a subgroup of the bHLH transcription superfamily, a family of transcription factors widely involved in developmental processes, ranging from neurogenesis to hematopoeisis and myogenesis.[3,4] As the subgroup and name suggests, this

receptor consists of a Helix-Loop-Helix (bHLH) domain and two PER-ARNT-SIM (PAS-A, PAS-B) domains. Of the available domains, bHLH was shown to be a DNA binding domain while the PAS-B domain was determined to be the ligand binding domain.[5] Likewise, the binding of Heat Shock Protein 90 (HSP90) during unliganded AHR conditions were found to be localized to both the bHLH and the PAS domains. Upon ligand binding, further research has shown the potential of HSP90 dissociation to play a role in AHR nuclear translocation.[6] Finally, a transcriptional activation domain was found to be located in the carboxy-terminal of this protein.

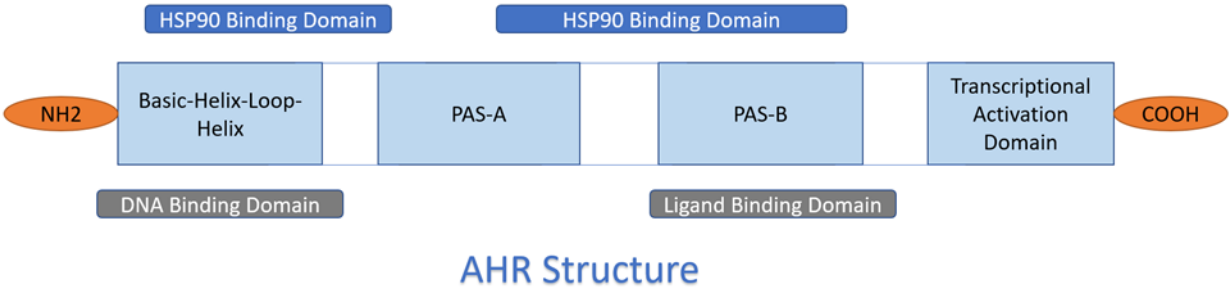

Figure 1: Functional Domains of AHR

Canonical Signaling Pathway for AHR

The mechanism of AHR has been shown to be constitutively active, indicative of a certain basal activity within the cells. As such, this receptor can be both upregulated and downregulated depending on environmental conditions. In its basal unliganded state, AHR has been shown to be present in the cytoplasm existing in complex with chaperone proteins, heat shock protein 90 (HSP90), co-chaperone p23, and X-associated protein 2 (XAP-2).[7] Upon ligand binding, an allosteric conformation takes place that leads to translocation into the nucleus. It has been postulated that the HSP90 chaperone proteins may potentially play a role in regulating nuclear

translocation.[7] Upon arrival in the nucleus, the chaperone complex dissociates from AHR upon association with its nuclear partner Ah receptor nuclear translocator (ARNT). Finally, this heterodimer complex is able to regulate transcription via binding to the genomic sequence Dioxin Response Elements (DRE) located upstream of genes such as *CYP1A1* and *AHRR*.[7] As such, activation of AHR has been shown to promote *CYP1A1* activity, a prominent phase 1 xenobiotic metabolizing enzyme, along with a myriad of other relevant enzymes associated with drug metabolism.[8]

Likewise, control of AHR has been shown to be regulated by a variety of negative feedback loops. Excess AHR levels may be controlled via proteasomal degradation via the ubiquitin-ligase complex.[7] Meanwhile, upregulation of *CYP1A1* due to AHR activation may also control for AHR activity via metabolism of viable ligands. Finally, excess AHR levels may also upregulate AHR Repressor (AHRR) levels.[9] The presence of the AHRR serves to modulate AHR activation via the binding of AHRR to ARNT in the nucleus, competitively inhibiting AHR binding and dimerization with ARNT.[9]

Depending on the cell line, it should also be noted that AHR may exhibit activation in a ligand independent manner. This was shown specifically in cells with relatively high levels of AHR in which dynamic nucleocytoplasmic shuttling make take place, enabling AHR and ARNT heterodimerization without need for ligand binding.[3]

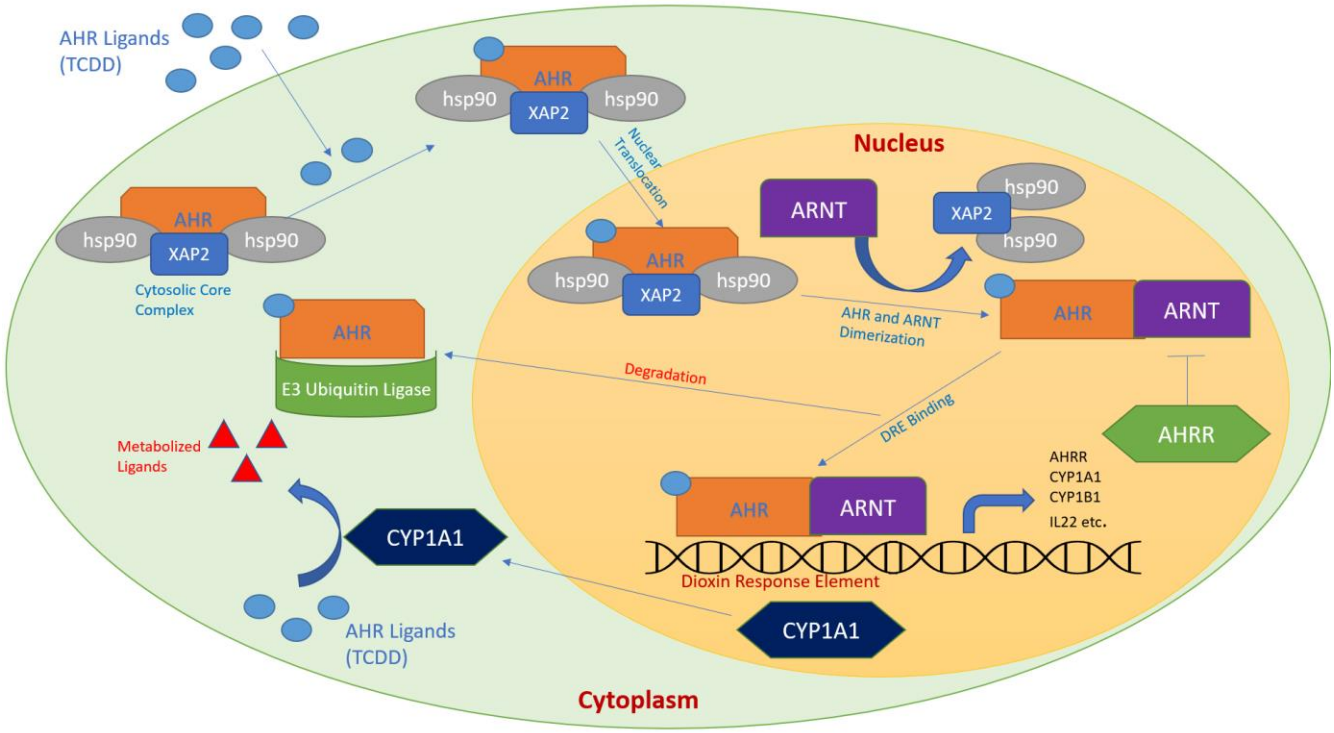

Figure 2: AHR Canonical Pathway Mechanism

Viable AHR ligands

As the only bHLH-PAS family members to bind and be activated by ligand, the AHR has been shown to be activated by a myriad of endogenous and exogenous ligands, from polycyclic aromatic hydrocarbons such as benzo (a) pyrene to persistent planar halogenated polycyclic hydrocarbons, most notably of which is 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD).[3] Depending on the tissue and ligand context, presence of endogenous ligands when compared to exogenous ligands may have contradictory effects on tumorigenesis.[3] Endogenous ligands generally consist of tryptophan metabolites such as kynurenine, indoxyl sulfate and 6-formylindolo[3,2b] carbazole (FICZ).[3] These endogenous ligands tend to activate AHR transiently, enabling a return to basal levels of AHR activity. Interestingly, studies have found

endogenous ligands in urine samples of healthy individuals that are considered to be benign or even beneficial. For example, observations correlating host mediated AHR activation to that of enhanced epithelial barrier function have been proposed.[3]

By contrast, exogenous ligands such as TCDD have been shown to facilitate significant levels of sustained activation via systemic circulation, resulting in adverse effects in the context of tumorigenesis. More specifically, TCDD has been shown to be an epigenetic carcinogen and potent tumor promoter functioning in the nanomolar range in rodents. As a tumor promotor, TCDD has been shown to induce T-regulatory cells, suppressor T cells that are believed to play a pivotal role in mediating the suppressed adaptive immune environment commonly found in tumors.[3] This is similarly linked to the ability of TCDD to induce AHR activity with recent studies also depicting AHR as a regulator of T cell differentiation. Interestingly, while AHR has been shown to induce both T regulatory and T Helper cell 17 (a pro-inflammatory Helper T-cell subset defined by Interleukin 17 secretion), TCDD tends to favor production of T-regulatory cells while an endogenous agonist such as 6-formylindolo[3,2b] carbazole (FICZ) was shown to favor T-Helper 17 cells, confirming a variance in functionality depending on the agonist and ligand used within a given study.[3]

In terms of origin, TCDD is formed as a byproduct of incomplete combustion with the release of TCDD often found during combustions of fossil fuels and incineration of industrial waste. Human studies as described by the Environmental Protection Agency have also related TCDD to soft tissue sarcomas, lymphomas, weakened immune systems and even chloracne, a severe acne condition.[10]

Interestingly, recent research has also identified dietary nutrients, in the form of various flavonoids, that were shown to exhibit AHR agonism and antagonism in a cell line specific

manner.[34] The most significant source of plant based AHR agonist activity is indolo glucosinolates, a compound abundantly found in cruciferous vegetables.[3] Upon metabolism, indolo glucosinolate is subsequently degraded into indole-3-carbinol. Under highly acidic conditions such as the stomach, this compound then undergoes condensation resulting in a myriad of AHR agonists, including the high AHR affinity indolo-[3,2 b]-carbazole (ICZ), a dietary ligand.[3]

AHR within a Cancer Context

Under the context of carcinogenesis, the effect of AHR activity has been shown to vary greatly depending on the cancer type and tissue of origin. For example, recent studies have suggested an attenuated tumor aggressiveness leading to a better prognosis when AHR is activated in hormone dependent breast cancers.[11] This inverse relationship between AHR activity and the histological grade of associated tumor was likewise attributed to the ability of AHR to antagonize the estrogen receptor.[3] By contrast, under the context of head, neck and lung cancers, elevated AHR activity have resulted in the opposite effect, leading to increased tumor aggressiveness and a poorer prognosis.[12]

In addition to variance, tumor invasion and potential for metastasis are vital aspects when looking at carcinogenesis and aggressive malignancies. The likelihood for a tumor to proliferate and invade is often regulated extensively by cell-to-cell contact and associated enzymes. Recent studies have shown increased AHR activity to be correlated with cell-cell contact deregulation and subsequent loss of E-cadherins.[13] Because E-cadherins are a family of calcium dependent transmembrane proteins responsible for adhesion, their loss from AHR activity may thus

promote greater cell migration.[13] The potential for tumor invasion and migration was further corroborated with a similar experiment performed in the head and neck cell lines; treatment with an AHR antagonist was shown to result in a significant reduction in the cells migration potential.[16] The association of the AHR with tumor invasion potential was further verified via studies on matrix metalloproteinase (MMP), a family of proteinases commonly upregulated in tumor cells with the purpose of degrading extracellular matrix proteins in allowing invasion to neighboring tissue. Analysis via quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) indicated a lower expression level of *MMP9* secretion when treated with antagonist for the HNSCC cell line.[16]

While there may be a high degree of variance and complexity involving the role of AHR activity in the context of cancer, the relevance of AHR and its role in tumorigenesis is nonetheless an important consideration when regarding therapeutic options.

Head and Neck Squamous Carcinoma

Head and Neck Squamous Cell Carcinoma (HNSCC) is a broad overarching heterogenous group of tumors commonly derived from the squamous epithelium of the oral cavity, oropharynx (mid-throat), hypopharynx (continuation of oropharynx) and the larynx. With 600,000 new cases per year, head and neck cancers have been found to be the 6th most common malignancy worldwide.[14] Historically, cancer has been accompanied by treatments ranging from surgery, radiation, and chemotherapy. A combination of all three treatments has often led to beneficial short term clinical outcomes. Yet despite this, HNSCC is still often associated with poor clinical outcomes, with 50% of patients suffering from relapse just 2 years later.[15] With

limited curative outcomes, 5-year survival rates for advanced stage HNSCC have not changed significantly at all in the past decades, lending an urgency for new therapeutic options at the molecular level.[15]

Previous studies of the HNSCC have revealed a role of the AHR in facilitating the aggressive phenotype of these cell lines.[16] More specifically, this was shown in the form of elevated interleukin 6 (*IL6*) and migratory potential. Interleukin 6 in particular is considered to be a major cytokine in the tumor environment, with its overexpression found in almost all cancer types.[17] This overexpression of *IL6* in HNSCC cell lines has been correspondingly linked to the high constitutive AHR activity in these cells. More specifically, the presence of AHR enables the *IL6* promotor to be found in a de-repressed state (thus greater accessibility of *IL6* promotor) due to displacement of the histone de-acetylase-1 co-repressor complexes.[18]

Overview of Research

Previous work from the Perdew lab have already depicted a role of AHR activity in enhancing an aggressive phenotype commonly seen in the HNSCC cell lines. The AHR exhibits a significant level of basal activity in these lines, thus we hypothesized that treating the head and neck lines with AHR antagonist would inhibit this aggressive phenotype. The HNSCC cell lines we use exhibit elevated basal AHR expression, with western-blots from the lab depicting HNSCC 30 (HN30) cells to have a 7-fold greater nuclear-AHR expression when compared to the Human Epithelia Keratinocyte (HEK) cell line.[16] With this higher basal AHR expression, it was suggested that antagonism of AHR activity would have a greater impact in HNSCC cell lines when compared to ordinary keratinocytes.[16]

The overall purpose of this thesis was to elucidate the role of AHR activation and inhibition as it relates to HSCSS cell phenotype and chemotherapy efficacy. In order to achieve this, experiments were done via cell culture and changes in cell survival were measured upon addition of various ligands in conjunction with chemotherapeutic treatment. More specifically, our expectations were to see an increase in susceptibility of chemotherapy under inhibited AHR conditions. As such, our goal was to antagonize AHR to further our understanding of whether AHR would be a viable drug target whose modulation may play a therapeutic role in cancer treatment.

To verify AHR activity, mRNA expression levels of *CYP1A1*, a downstream target of AHR, was calculated under known AHR agonist (TCDD) and antagonist (IK10364) conditions. A dose of 5-fluorouracil (5-FU) that leads to 50% cell viability (LD_{50}) was utilized and the effect of AHR antagonist (IK10364) was assessed. Cell proliferation and cell viability were measured quantitatively via spectroscopy and colony forming assays.

Materials and Methods

Cell Culture

The HNSCC Cell line 30 (HN30) was maintained at 37 °C, 5% CO2 in a high glucose 1:1 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12) medium from Sigma Aldrich. The medium was modified with the addition of 1000 units of Penicillin and 0.1 mg/ml streptomycin (final concentration) obtained from Sigma along with 8% Fetal Bovine Serum (FBS) purchased from Hyclone Laboratories. The HN30 Cell line was maintained and split approximately every 3-4 days into a 100 mm Polystyrene Tissue Culture Treated Dish. Phosphate Buffered Saline (PBS) Wash and 1X Trypsin-EDTA solution were used for passaging the cells.

Chemical Reagents

TCDD was provided by Dr. Steve Safe of Texas A/M University. To maintain physiological relevance, TCDD treatments were performed at approximately 2 nanomolar (nM) concentration. Likewise, the IK10364 AHR antagonist, a new compound provided by Ikena Oncology was also utilized in the nanomolar range.

The 5-Fluorouracil (5FU) was obtained via Sigma and was prepared by dissolving in 1 N ammonium hydroxide. The 5FU solution was filtered through a Nalgene 0.2 µm surfactant free acetate membrane (SCFA) before being used for treatment in media. Similarly, the 0.5% methylene blue dye was also filtered through SCFA before being used for the colony forming assay.

RNA Isolation and Quantitative Reverse Transcription PCR

TRIzol reagent (Sigma-Aldrich, St. Louis, MO) was used to isolate RNA after specified treatment. Addition of chloroform after TRIzol addition separated the reagent into aqueous and organic phases with the RNA in the aqueous phase. The RNA was then recovered via precipitation by isopropyl alcohol. 70% ethanol was then used to wash the sample before it was maintained in warm DEPC water. Details about the TRI reagent procedure were also specified by the manufacturer.[19] Upon successful isolation, RNA concentrations were measured via NanoDrop Spectrophotometer (ThermoFisher Scientific).

Isolated RNA was then converted to cDNA via an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. mRNA levels were finally measured via quantitative reverse transcription PCR (qRT-PCR). Reagents were prepared using the PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA). A CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was implemented with the iCycler DNA engine. Genes were normalized via glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *B-ACTIN* for activity. Associated primers used for reverse transcription PCR have been listed in the appendix as Table 1.

Cell Counting Kit 8

Cell Counting Kit 8 (CCK8) was a viability assay used to accurately measure cell number and proliferation within a given well. This assay utilizes a highly soluble tetrazolium salt, WST-8, which upon reduction by an electron mediator, produces an orange-colored water-soluble

formazan dye. The amount of formazan was directly associated to the number of living cells and subsequently quantized via spectrophotometry.[20]

The CCK8 Viability Assay was used for both a 5-fluorouracil (5FU) kill curve and an antagonist susceptibility procedure to a standardized 5FU dosage. In both cases, 4000 cells per well were seeded out in a 96 well plate and 10 ul of CCK8 reagent were injected into the 200 ul media after the specified treatment period. A media control plate was also maintained, and both the experimental and control plates were covered with aluminum foil and allowed to incubate at 37 °C for 4 h before being read by spectrophotometry.

Colony Forming Assay

Colony Forming Assay was done by seeding 120,000 cells per well into 2 6-well plates. After a specified treatment period, these cells were then detached via accutase, a gentler trypsin replacement, before they were reseeded into 6-well plates as triplicates with 400 cells per well. The colonies were then left to incubate in 37 °C for 8 days until they were visible to the naked eye. The plates were then imaged and washed via 0.5% methylene blue dye and nanopure water.

Statistical Analysis

Raw Data was analyzed using Prism 7 graphing and statistical analysis software (GraphPad Software inc). Outliers were generally excluded during the characterization of data. qPCR data was further analyzed using a one-way ANOVA (Analysis of Variance). Data was considered statistically significant with a p-value ≤ 0.05 and categorized as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P<0.0001$.

Cell Viability and Colony forming assays were analyzed via relative area when compared to a vehicle control (no treatment) and visualized in a column bar-graph format. ImageJ (FIJI) was used to set an optimal threshold for methylene blue colony detection; area was then analyzed according to the given threshold **(Figure 3)**. Triplicates were ensured for each sample of the colony forming assay. Finally, relative area when compared to the control was calculated by dividing individual triplicates by the average of vehicle (no treatment) area values.

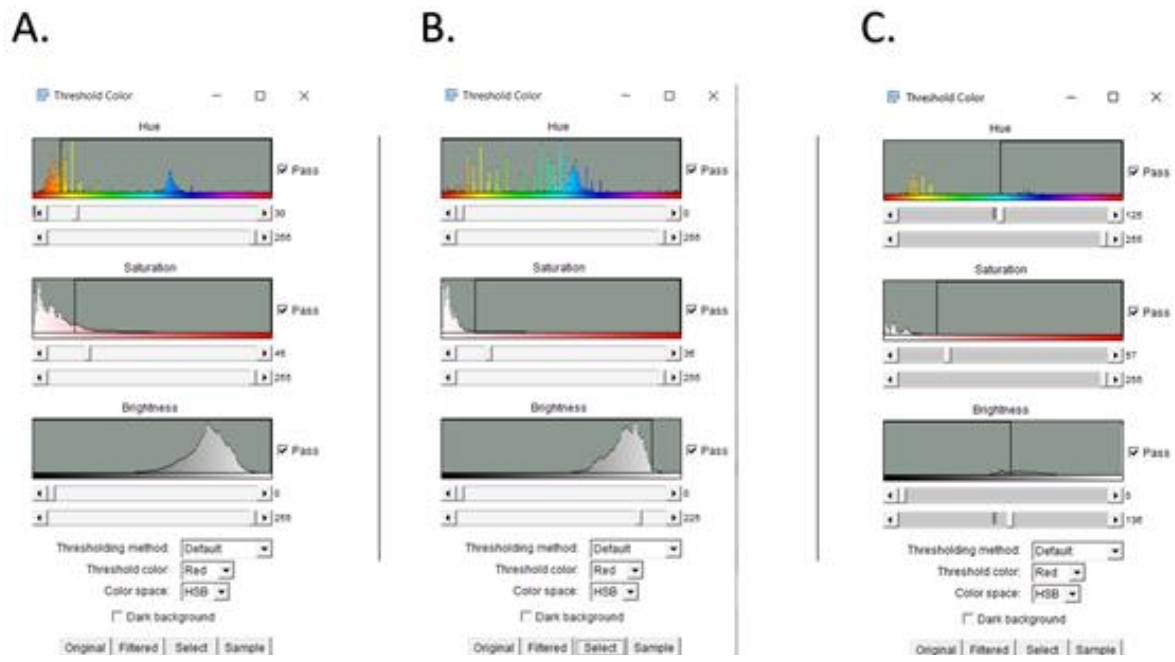

Figure 3: Color Threshold Values for ImageJ

*Color Threshold Statistics for 5FU treated **(A)**, and no 5FU treated **(B)** colony forming assay with variable IK10364 antagonist concentrations. Threshold statistics were also done for 2nM TCDD treated and untreated colony forming assay with variable 5FU dosage **(C)**.*

Results

The Perdew Lab had previously characterized the HN30 cell line to have high constitutive AHR activity compared to other head and neck tumor cell lines. In addition, treatments of antagonist N- [2-(3H-indol-3-yl)ethyl]-9-isopropyl-2-(5-methyl-3-pyridyl)purin-6-amine (GNF351) and trimethoxyflavone (TMF) were also previously found by the Perdew laboratory to inhibit proliferation, invasion and migratory potential.[16] Antagonism of the AHR within the HN30 cells were overall found to have decreased aggressive phenotype within the HNSCC cell line.[16] Here, we attempt to characterize tumorigenesis from a different perspective. To do so, we looked at associated carcinogenesis factors under context of TCDD, a potent AHR agonist. In addition, a novel antagonist IK10364 was further tested for chemotherapy susceptibility upon treatment to the HN30 cell line.

AHR Agonist TCDD Upregulates Relative mRNA Expression of Cancer-Related Targets

TCDD is an exogenous ligand commonly characterized to be a potent AHR agonist.[3] To verify the integrity of our TCDD sample, 2nM TCDD treatment of the HN30 cell line for 72 h was performed and compared with a dimethyl sulfoxide (DMSO) solvent vehicle. Subsequent qRT-PCR of *CYP1A1*, a target downstream of AHR, showed a statistically significant rise (~160 Fold Change) in *CYP1A1* relative mRNA levels when compared to the vehicle control, confirming TCDD's role in inducing AHR **(Figure 4A)**.

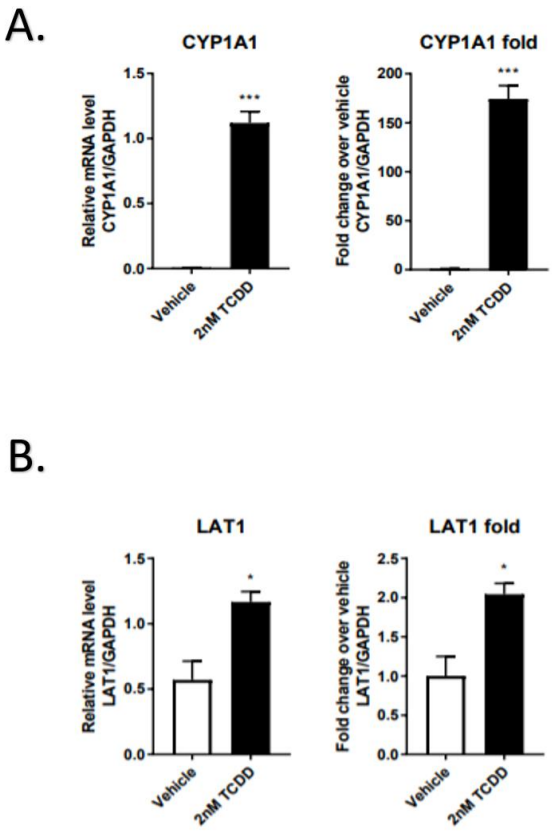

Figure 4: Relative mRNA expression levels of *CYP1A1* and *LAT1*

HN30 cells were treated with either TCDD or DMSO (Vehicle) to achieve a final concentration of 2nM. RNA Isolation, cDNA generation and qRT-PCR were performed for both CYP1A1 ***(A)*** *and LAT1* ***(B)*** *for relative mRNA expression and fold change. Expression levels were normalized using GAPDH and asterisks indicate statistical significance: P < 0.05 (*); P < 0.01 (**); P < 0.001(***); P<0.0001 (****).*

Various transporters closely associated with tumorigenesis were also characterized via qRT-PCR. Specifically, the L-type amino acid transporter 1 (*LAT1*) is a heterodimeric membrane transport protein notorious for transporting large neutral amino acids (leucine, isoleucine, valine etc).[21] With its role in nutrient uptake, LAT1 is a target that has been significantly correlated to proliferation and angiogenesis.[21] Likewise, studies have shown LAT1 to be overexpressed in numerous cancer types, most notably that of non-small cell lung cancers, for which overexpression was shown as a marker of poor prognosis.[22,23] To further elucidate *LAT1* mRNA expression in the context of AHR activity, HN30 cells were treated with TCDD for 72 h before

RNA isolation, cDNA generation and subsequent qRT-PCR was done for *LAT1* mRNA levels. Results indicated a statistically significant ($P<0.05$) increase in *LAT1* expression levels for TCDD treated samples when compared to vehicle **(Figure 4B)**.

Similarly, ATP-binding cassette sub-family G member 2 (*ABCG2*) is a member of the ATP-binding cassette (ABC) transporter superfamily. Alternatively coined as the Breast Cancer Resistance Protein (BCRP), this transporter is specifically known to be a xenobiotic transporter.[24] As such, studies have shown involvement of *ABCG2* drug efflux to be a prominent source of chemotherapy treatment resistance and failure.[25] TCDD treatment of HN30 cells for 72 h and subsequent qRT-PCR likewise also resulted in a statistically significant ($P<0.01$) increase in *ABCG2* expression levels when compared to the control **(Figure 5D)**.

Additionally, sestrin-2 (*SESN2*), is a stress inducible protein that has been reported to play a critical role in regulation of nutrients and survival for glutamine depleted cancer cells.[26] As such, in a tumor environment where there may be a limited availability of resources, the ability of sesetrin-2 to regulate cell growth and survival may have an even greater impact when viewed in the context of carcinogenesis. Experimentally, 2nM TCDD treatment of HN30 cells for 72 h had revealed a statistically significant ($P<0.01$) increase in relative mRNA expression levels when compared to vehicle **(Figure 5C)**.

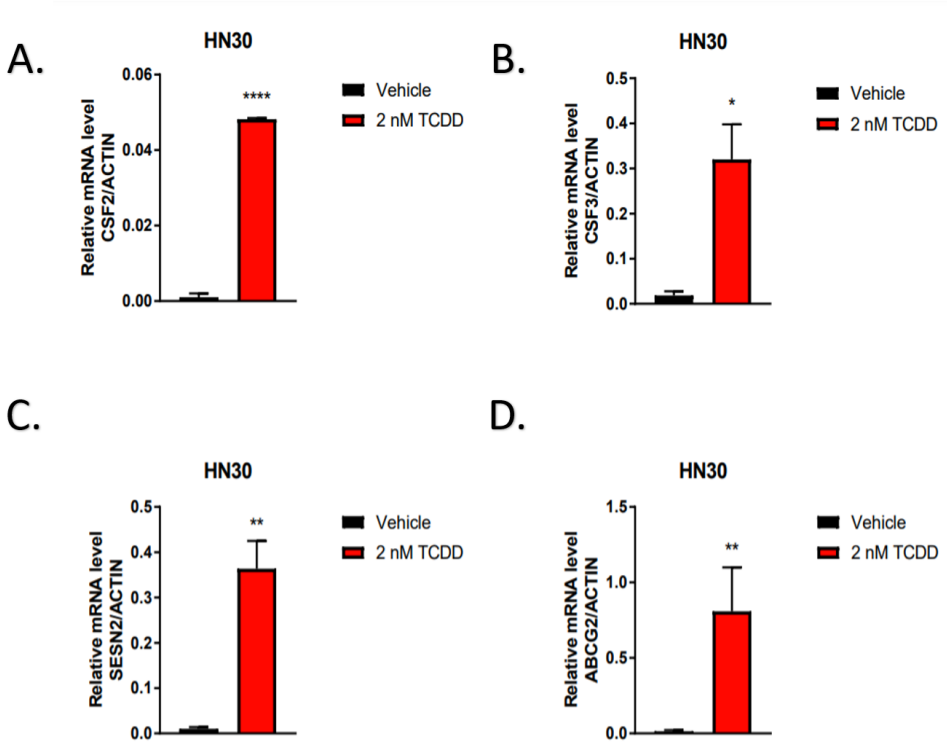

Figure 5: Relative mRNA expression of *CSF2, CSF3, SESN2,* and *ABCG2*

HN30 cells were treated with either TCDD or DMSO (Vehicle) to achieve a final concentration of 2nM. RNA Isolation, cDNA generation and qRT-PCR were performed for CSF2 ***(A)****, CSF3* ***(B)****, SESN2* ***(C)****, ABCG2* ***(D)*** *for relative mRNA expression. Expression levels were normalized using ACTIN and asterisks indicate statistical significance: P < 0.05 (*); P < 0.01 (**); P < 0.001(***); P<0.0001 (****).*

In addition, several cytokines were also characterized by association with AHR inducement. More specifically, Granulocyte-Macrophage Colony Stimulating Factor (*CSF2*) and Granulocyte Colony Stimulating Factor (*CSF3*) were characterized for mRNA expression levels under 2nM TCDD treatment. Both *CSF2* and *CSF3* have been shown to stimulate production granulocytes, a category of white blood cells, indicating a potential role in the tumor microenvironment via recruitment of immune cells.[27]

Similarly, interleukin cytokines were also looked at for their inflammatory properties. The significance of inflammation within a tumor microenvironment have already been well

documented in previous studies and often found critical in all steps of tumor progression, including that of metastasis.[28] Both Interleukin 1β (IL1β) and Interleukin 6 (IL6) are pro-inflammatory cytokines with recent studies depicting IL1β as pro-tumorigenic, with IL1β having been found to promote the spheroid forming capabilities of colon cancer cells.[29] Similarly, IL6 is known to be deregulated in cancer, with its overexpression reported in all types of tumors.[30] Studies have found IL6 to play a role in protecting cancer cells from therapy induced DNA damage, apoptosis and oxidative stress.[17,30] The association of IL6 and IL1β to AHR activity was as such similarly characterized via 2nM TCDD treatment for 72 h and subsequent qRT-PCR for the targets of interest. A statistically significant increase of relative mRNA expressions in both IL1β ($P<0.001$) and IL-6 ($P<0.01$) were found upon analysis **(Figure 6 A-B)**.

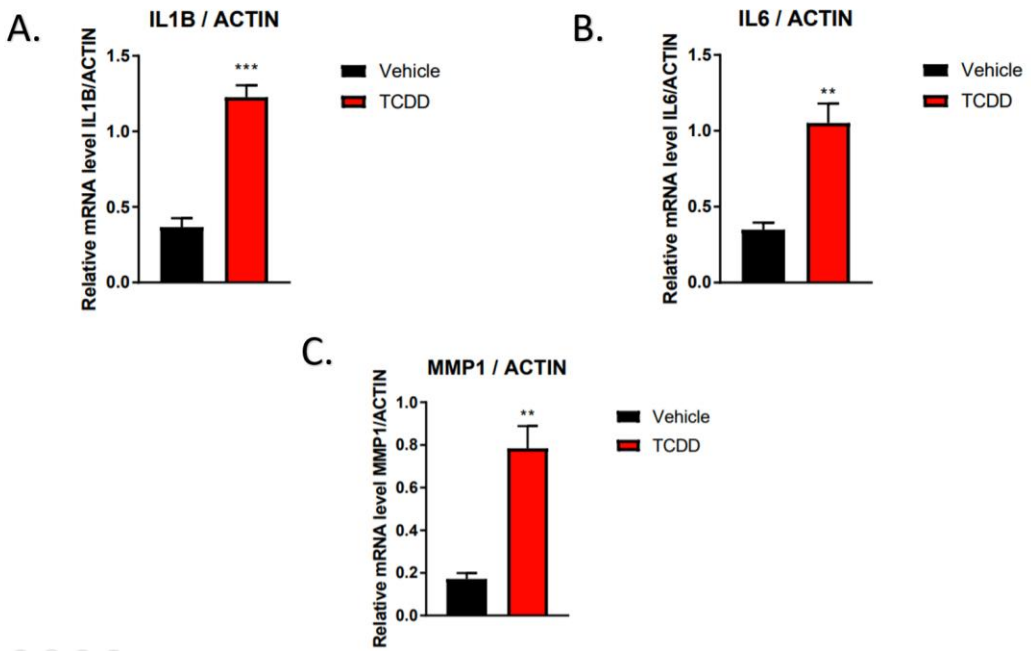

Figure 6: Relative mRNA expression of *IL1B*, *IL6*, and *MMP1*

*HN30 cells were treated with either TCDD or DMSO (Vehicle) to achieve final concentrations of 2nM. RNA Isolation, cDNA generation and qRT-PCR were performed for IL1B **(A)**, IL6 **(B)**, and MMP1 **(C)** for relative mRNA expression. Expression levels were normalized using ACTIN and asterisks indicate statistical significance: $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$(***); $P<0.0001$ (****).*

Finally, Matrix Metalloproteinases (MMP) are a family of proteinases commonly known for their role in extracellular matrix protein degradation.[31] Commonly secreted by tumors, this degradation of the extracellular matrix would likely further promote metastasis and invasion into neighboring tissue.[16] The Perdew lab had previously characterized the role of AHR in active secretions of MMP9 via gelatin zymography.[16] In addition, antagonism studies were also done, with lower levels of *MMP9* associated with AHR antagonist treated HN13 cells.[16] Further characterization as such were performed with MMP1. While MMP9 was known to degrade collagen IV and V, MMP1 alternatively focuses on Collagen I, II, III degradation.[32,33] 2nM TCDD treatment of HN30 cells for 72 h as such revealed a statistically significant increase in *MMP1* levels upon associated inducement of AHR by TCDD **(Figure 6C)**.

New Antagonist IK10364 Downregulated AHR in a Dose-Response Manner

Keeping in mind previous experiments of the Perdew lab regarding AHR antagonists, a novel antagonist classified as IK10364 by Ikena Oncology was utilized. To characterize IK10364 efficacy in inhibiting AHR activity, HN30 cells were treated with the novel antagonist for a period of 24 h before relative mRNA expression levels of *CYP1A1* were quantized. This data was then analyzed in comparison to the vehicle mediated *CYP1A1* mRNA levels. A dose-dependent relationship between IK10364 concentration and percentage of relative mRNA expression compared to vehicle was observed, with a statistically significant decrease in percentage of vehicle mRNA levels as antagonist concentrations increased **(Figure 7A)**. To verify this data and in turn functionality of the AHR receptor, relative *CYP1A1* mRNA levels

were also measured after 24 h TCDD treatment. A significant increase of relative expression in the TCDD treated group was noticed when compared to the vehicle control **(Figure 7B)**.

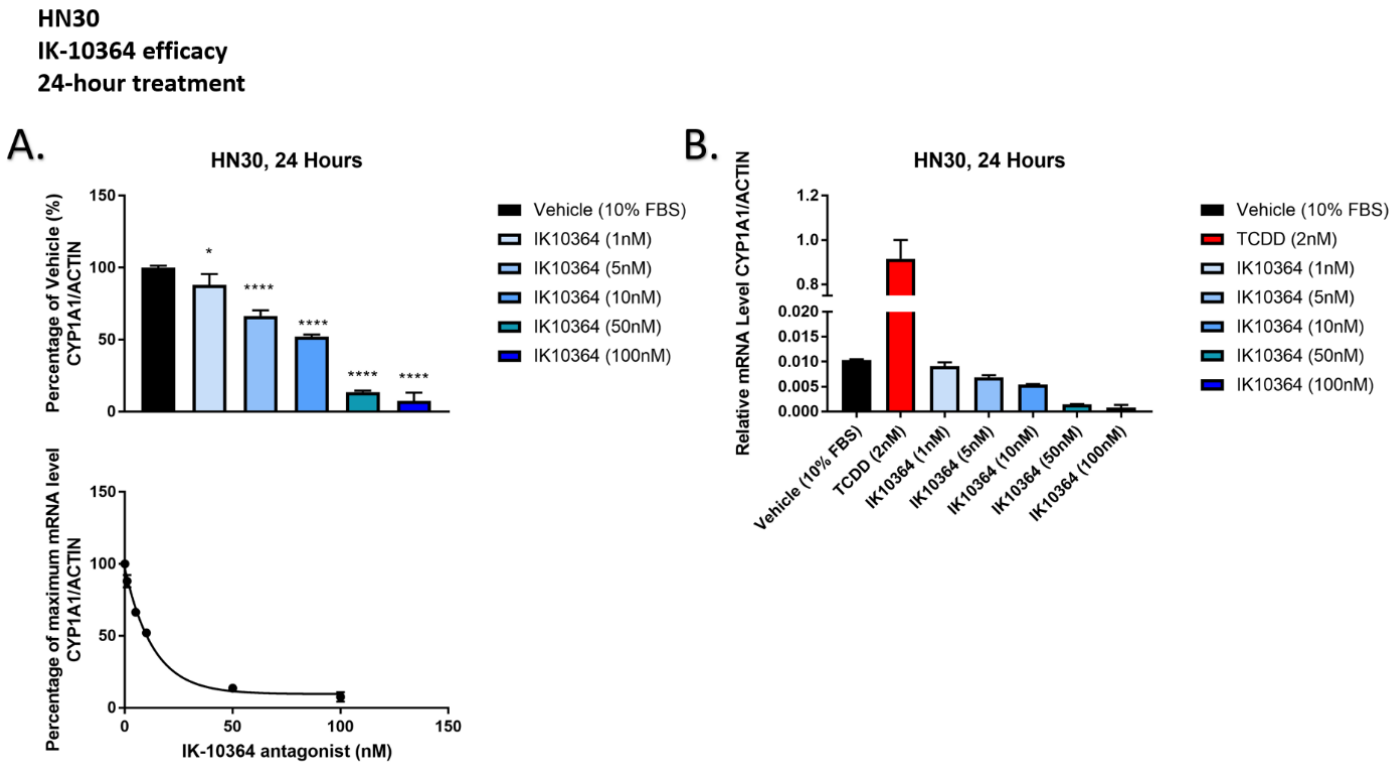

Figure 7: Relative mRNA Expression levels for CYP1A1 under IK10364 and TCDD treated conditions

*HN30 cells were treated with either TCDD, DMSO (vehicle), or IK10364 antagonist. The antagonist IK10364 was done in a gradient of increasing final concentrations (1nM, 5nM, 10nM, 50nM, 100nM). RNA Isolation, cDNA generation and qRT-PCR were done for CYP1A1. Expression levels were normalized using ACTIN and relative mRNA expression was calculated as a ratio of the vehicle. Asterisks indicate statistical significance: P < 0.05 (*); P < 0.01 (**); P < 0.001(***); P<0.0001 (****). Data was analyzed first without incorporation of TCDD to measure statistical significance in a column and line graph visualization **(A)**. TCDD was then incorporated into the data as a positive control for AHR induction **(B)**.*

Time and Concentration was Found via 5FU Kill Curve

With confirmation of antagonist efficacy, susceptibility of HN30 cells to chemotherapy was then characterized by treatment of HN30 cells with variable dosage of 5-fluorouracil (5FU) for a period of both 24 and 48 h. Cell efficacy and survival was then analyzed via CCK8 viability assay; the results were graphed according to a percentage of viable cells normalized against the vehicle control (no 5FU). Between the 24 and 48 h, the 48 h graph was found to be the most well defined. As such, the 48 h timepoint was used to formulate a standardized curve for generation of a median lethal dose (LD_{50}). The LD_{50} was determined to be approximately 0.6 ug/ml **(Figure 8B)**.

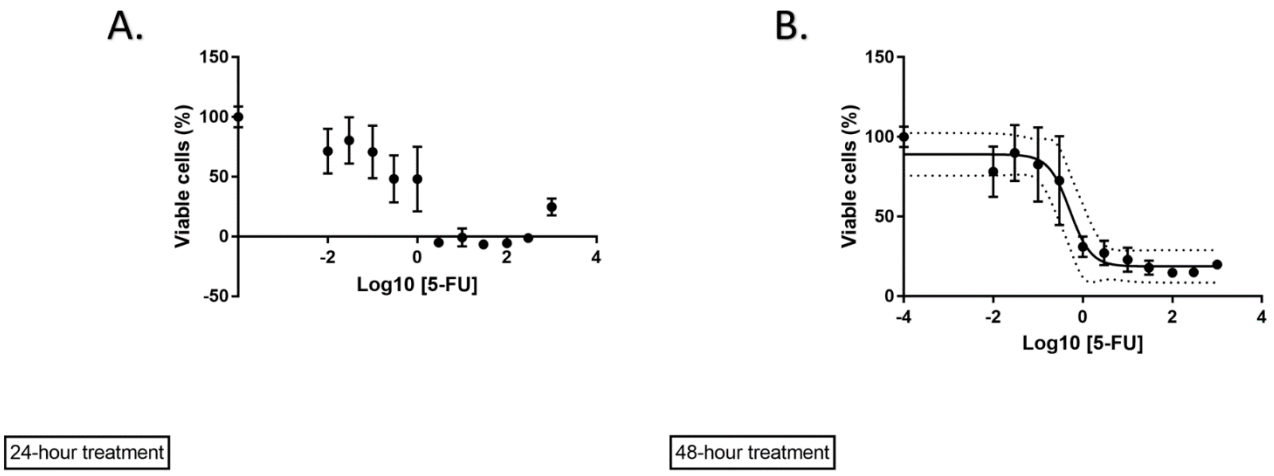

Figure 8: 5FU Kill Curve for HN30 cells 24, 48 h

*HN30 cells were seeded 4000 cells per well in a 96 well plate. Cells were treated with variable 5FU (1000ug/ml, 300ug/ml, 100ug/ml, 30ug/ml, 10ug/ml, 3ug/ml, 1ug/ml, 0.3ug/ml, 0.1ug/ml, 0.03ug/ml, 0.01ug/ml, 0ug/ml) total concentrations. 5FU treatment of HN30 cells was done for both a 24 h **(A)** and 48 h **(B)** time period. Percent cell viability was measured via the CCK8 kit and analyses were done via Graphpad Prism 7. The 48 h 5FU kill curve was ultimately used for further quantification and the LD_{50} was found to be approximately 0.6ug/ml.*

AHR Antagonist IK10364 decreased susceptibility to 5FU

Upon quantification of an LD_{50}, susceptibility of HN30 cells to chemotherapy was tested under modulation of AHR activity via inhibition with the new antagonist IK10364. HN30 cells were pretreated with IK10364 for 24 h (based on previous qRT-PCR data) and subsequently treated with a fixed 5FU LD_{50} dosage of 0.6 ug/ml for a period of 48 h (as determined by the 5FU kill curve). Susceptibility to the 5FU chemotherapy was found to decrease substantially with addition of the new IK10364 treatment. This decrease in susceptibility was found to be in a dose-dependent manner, decreasing more as antagonist concentrations increased **(Figure 9)**.

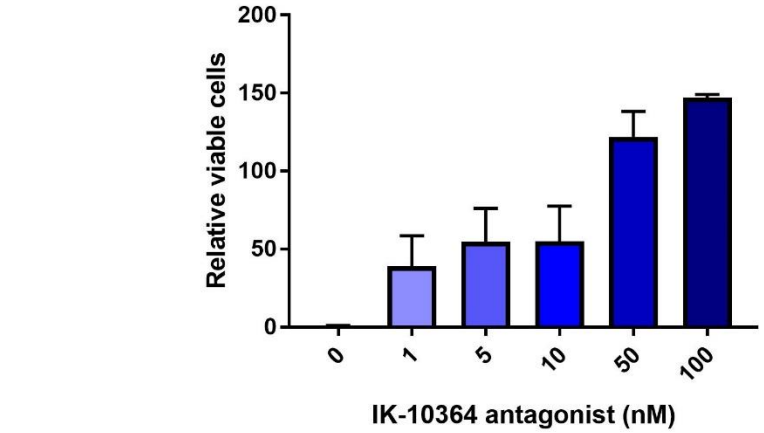

Figure 9: HN30 Cell Viability after Chemotherapy with Variable 5FU treatment

HN30 cells were seeded 4000 cells per well in a 96 well plate. Cells were pretreated with variable IK10364 antagonist (0nM, 1nM, 5nM, 10nM, 50nM, 100nM)) total concentrations for 24 h before treatment with 0.6ug/ml 5FU for 48 h. Relative viable cells were analyzed via CCK8 Kit and visualized via Graphpad Prism 7.

Colony Forming Assay Verified Decreased Susceptibility to 5FU from IK10364

To verify the effect of the IK10364 antagonist in decreasing chemotherapy susceptibility, a colony forming assay was done to measure overall cell health and survivability under antagonist and 5FU conditions. Cells were pretreated with variable concentrations of IK10364 for 24 h, before a standardized LD_{50} 5FU treatment of 0.6 ug/ml was added for 48 h. A significant increase in colony relative area was seen in cells grown under high 100 nM antagonist treatments after exposure to 5FU **(Figure 10)**.

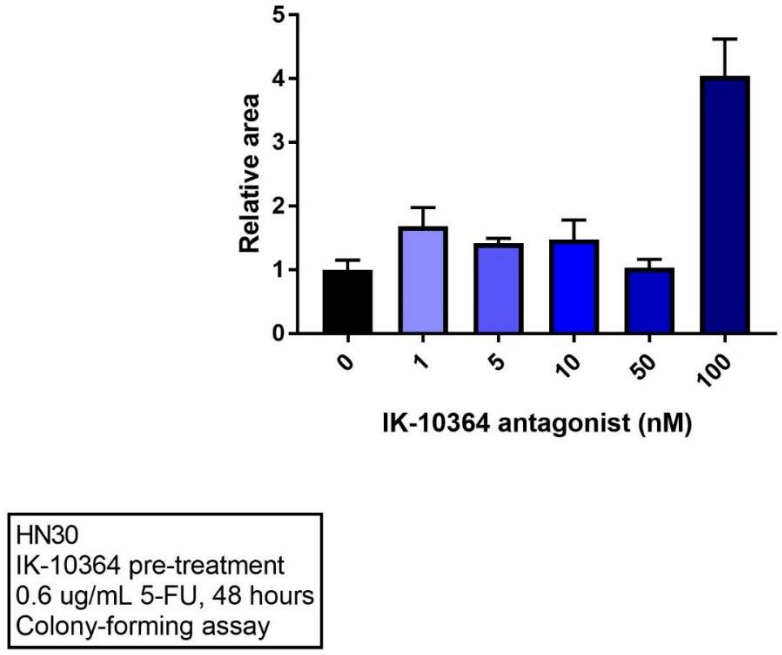

Figure 10: Colony Forming Assay after 24 h Antagonist Pretreatment and 48 h 5FU Exposure

HN30 cells were pretreated for 24 h with variable IK10364 (0nM, 1nM, 5nM, 10nM, 50nM, 100nM) final concentrations before being exposed to 0.6 ug/ml 5FU chemotherapy for 48 h. Each antagonist dosage was then split into 6 well plates as triplicates at 400 cells/well before they were left to grow. 0.5% methylene blue dye was used once colonies were visible and relative area was measured via ImageJ. Data was normalized as a ratio of the average area of vehicle control.

Finally, to ensure that antagonist IK-10364 itself did not directly impact cell phenotype, a colony forming assay consisting of just variable IK10364 treatments was done to verify consistency of colony growth. Though there was one outlier, not a significant difference in relative area was noticed upon increasing the dosage of IK10364 antagonist under no 5FU conditions **(Figure 11)**.

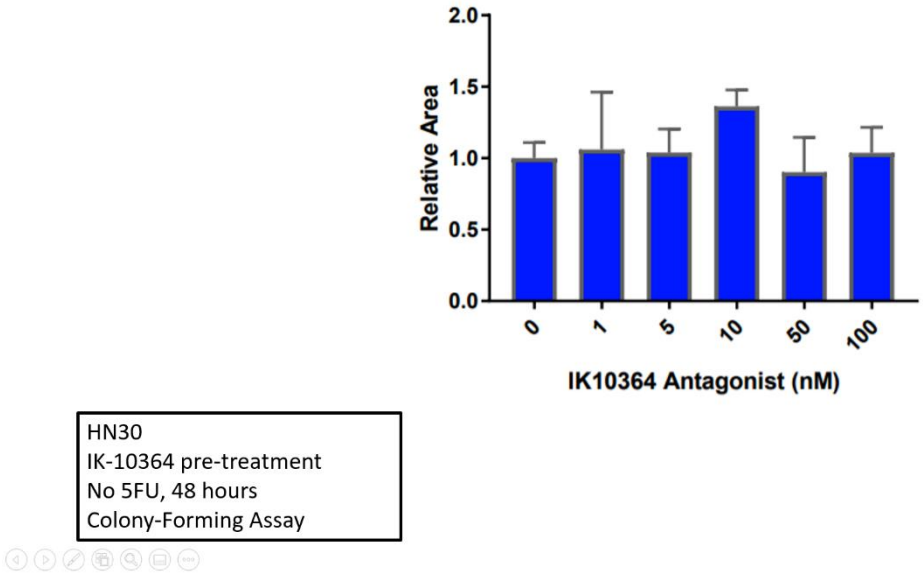

Figure 11: Colony Forming Assay after 24 h Antagonist Pretreatment and No 5FU exposure for 48 h

HN30 cells were pretreated for 24 h with variable IK10364 (0nM, 1nM, 5nM, 10nM, 50nM, 100nM) final concentrations before being incubated at 37 °C for 48 h. Each antagonist dosage was then split into 6 well plates as triplicates at 400 cells/well before they were left to grow. 0.5% methylene blue dye was used once colonies were visible and relative area was measured via ImageJ. Data was normalized as a ratio of the average area of vehicle control.

Colony Forming Assay indicated elevated susceptibility to 5FU with TCDD

As a supplemental experiment, HN30 cells were subjected to a variable 5FU chemotherapy given a pretreatment of either TCDD, an AHR agonist, or DMSO (the vehicle control). Interestingly, upon analysis of the colony forming assay, cells pretreated with TCDD were found to be overall less healthy and more susceptible to chemotherapy. This was illustrated by a remarkable drop in relative area between the 0.78 ug/ml and 1.56 ug/ml for TCDD treated cells **(Figure 12B)**. Meanwhile, a higher concentration of 5FU was required to show that same drop in relative area for vehicle treated cells with a majority of cells dying around the 1.56 ug/ml and 3.125 ug/ml range **(Figure 12A)**.

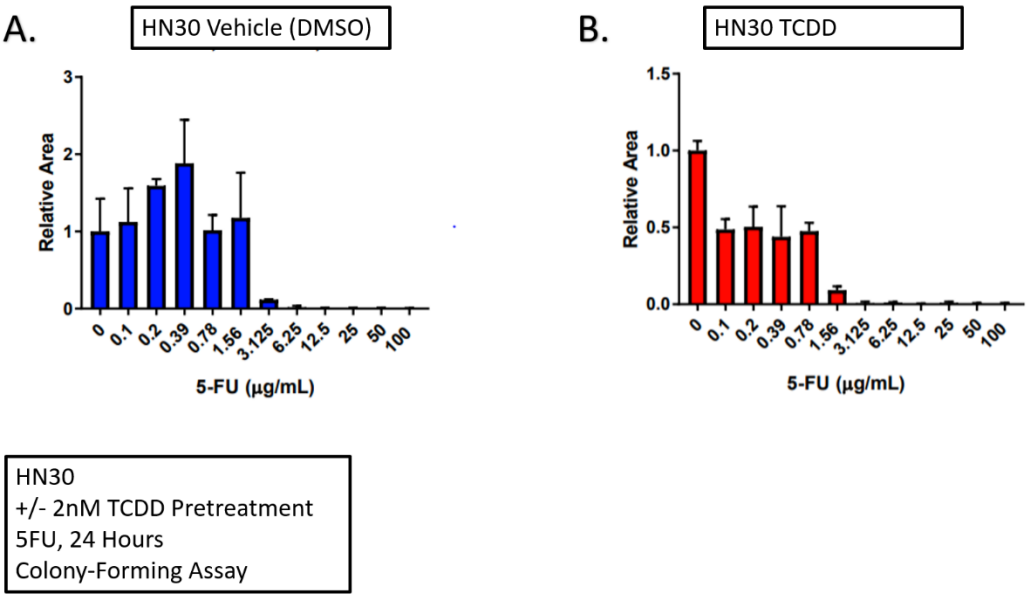

Figure 12: Colony Forming Assay +/- TCDD pretreatment with variable 5FU exposure

*HN30 cells were pretreated for 24 h with TCDD **(A)** or DMSO **(B)** at 2nM total concentration before being exposed to 5FU for 24 h. Each antagonist dosage was then split into 6 well plates as triplicates at 400 cells/well before they were left to grow. 0.5% methylene blue dye was used once colonies were visible and relative area was measured via ImageJ. Data was normalized as a ratio of the average area of vehicle control (0nM IK10364).*

Discussion

This thesis aimed to evaluate the impact of AHR modulation on overall HNSCC survival. Previous studies by the Perdew lab had identified a role of AHR antagonism in attenuating HNSCC aggressive phenotype via reduction of invasion and migratory potential.[16] As such, we hypothesized that the antagonism of AHR would promote chemotherapy susceptibility of HNSCC cell line via repression of the HNSCC aggressive phenotype. To evaluate the modulation of AHR on HNSCC, both TCDD and the novel antagonist IK10364 was used. IK10364 and TCDD were confirmed to respectively inhibit and induce AHR activity via qRT-PCR of downstream target *CYP1A1* and both were found to be statistically significant. However, while the relative *CYP1A1* mRNA expressions were as expected, assays used to measure cell survivability yielded results that did not match our hypothesis. Both the cell viability assay and the colony forming assay of IK10364 treated colony yielded data that indicated a decrease in susceptibility to chemotherapy in a dose-response manner. Similarly, colony forming assay of TCDD treated cell colonies revealed an increase in susceptibility to chemotherapy. While unexpected, the data further emphasized the complexity of the tumor microenvironment.

To supplement the results, cancer-related targets were additionally characterized under the context of TCDD sustained AHR activation via qRT-PCR. Of the targets that were analyzed, virtually all played a role in tumorigenesis and the tumor microenvironment. In addition, upon treatment of 2nM TCDD, a known AHR agonist, all associated targets exhibited a statistically significant increase in mRNA expression levels. The myriad of targets associated with the induction of AHR likewise highlights the complexity of AHR as a possible mediator of

numerous pathways that may promote overall HNSCC aggressive phenotype. While the upregulation of these cancer-related targets all seemed to suggest pro-carcinogenesis behavior, it is interesting to note the multifaceted functionality of these targets. For example, *CSF2* and *CSF3* were both cytokines characterized as pro-inflammatory, a characteristic hallmark of carcinogenesis.[28,35] However, *CSF2* has also been found to be an adjuvant in Dendritic Cell (DC) recruitment and subsequent presentation of tumor antigens to T-cells.[36] Thus, while *CSF2* inflammation has been shown to be a hallmark of carcinogenesis, it has also been found to promote anti-tumor immunity and delayed tumor growth via Interleukin-9- producing T helper (Th9) cells that secrete cytotoxic T-cell lymphocytes (CTL).[36] Coincidentally, qRT-PCR analysis of *CSF2* was found to be the most statistically significant ($P<0.0001$) **(Figure 5A)**.

Consideration of Context

To fully understand the given data, special notice should also be given to the nature of this research in taking a pharmacological approach to inhibition. While in many cases, the method of a receptor knockdown and receptor inhibition tend to be attributed to the same phenotype, the basis of these two methodologies are very different, with receptor knockdown focusing on the DNA level, while pharmacological inhibition targets the protein level.[37] As such, there is a distinct difference between pharmacological inhibition and knockdown experiments, with said differences often due to off target effects and specific protein-protein interactions.[37] More specifically, a complex treated with a pharmacological inhibitor, while inhibited, could theoretically still act as a physical scaffold for protein-protein interactions vital for biological function.[37] A knockdown at the DNA level however would ensure that there would no longer be

any protein present, inhibiting both the protein and off target scaffolding effects.[37] As such, it is likely that the modulation of AHR via pharmacological inhibitors and activators may lead to off target effects.

With this in consideration, a possible explanation for the differing phenotypes given expected relative mRNA levels could be that differing AHR agonists and antagonists are capable of altering their own separate subset of genes, thus enabling two separate pathways to one specific phenotype.[16] Previous studies have already found TCDD induced AHR to modulate the retinoblastoma (Rb) protein, positively regulating cell cycle via a direct interaction with phosphorylated Rb.[38] Interestingly, TCDD induced AHR activation has also been found to inhibit cyclin dependent kinase 2, negatively impacting cell cycle regulation and proliferation.[39] Other gain of function studies implemented revealed the inducement of tumors via sustained constitutive AHR activation in transgenic mice, furthering the complexity of AHR as a context dependent cell cycle regulator.[40] Similarly, TCDD has also been shown to play a role in phosphorylating the epidermal growth factor receptor (EGFR), replicating EGFR ligand binding conditions, thus enabling cells to have characteristics resembling an excess of epidermal growth factors.[41] The diversity of effects for TCDD induced AHR activation alone demonstrates the complexity of the tumor microenvironment and AHR's role in both positively and negatively regulating cell cycle and phenotype.

Additionally, while excessive proliferation is a common marker of tumorigenesis, overall cell survival as a balance between cell death and growth possess as great, if not even greater role.[42] Carcinogenesis as such, can be seen to be context dependent with multiple factors in play with each other. Another consideration to keep in mind is the heterogeneity of the population. From a therapeutics perspective, the presence of intratumor heterogeneity, a characteristic of

multiple subpopulations within a single tumor, poses another challenge to cancer treatment as targeted therapeutics are often cell line specific.[43]

Limitations of Monolayer Cell Culture

In addition to the complexity of the tumor microenvironment, limitations should also be accounted regarding the monolayer culture system implemented. While monolayer cell culture is still widely used due to its availability and reproducibility in vitro, the application the results may have is often limited due to large differences from the in vivo state.[44] Cells in most organisms tend to live in complex 3-dimensional states surrounded by both an extracellular matrix (ECM) and a diverse array of other cells.[45] Cell morphology and the diversity of cell-cell and cell-ECM interactions within a monolayer culture as such is vastly different from real life conditions.[44] More specifically, tumor themselves consist of a complex microenvironment and often contain complex phenotypes such as nutrient deprived conditions, hypoxia and vasculature not often replicated within a monolayer culture.[44,46]

Lack of interactions with the extracellular matrix and more specifically fibroblasts within the monolayer culture system is an additional limitation that should be considered in the context of tumorigenesis. Fibroblasts are traditionally known for their role in the synthesis and modification of the extracellular matrix (ECM). Under normal conditions, fibroblasts tend to be activated in instances of wound healing in order to rebuild and remodel the ECM.[47] However, in the case of cancer, constant assault leads to sustained aberrant fibroblastic activation and hence prolonged ECM remodeling.[47] More specifically, recent studies have shown a crosstalk between

fibroblasts and various inflammatory cytokines, chemokines and growth factors with aberrant activated fibroblasts leading to chronic inflammation.[48]

Additionally, activated fibroblasts tend to also be recognized by cells in the tumor microenvironment that provide metabolic support for cancer cells under conditions of nutrient deprivation further promoting tumorigenesis.[47] The functionality of fibroblasts within the tumor microenvironment as such provides an additional complication that was not accounted for via the monolayer culturing system.

Future Implications

Despite the complexity and limitations however, it is nevertheless undeniable the implications AHR has as an effective drug target for therapeutics in cancer. In addition to AHR's role in modifying conventional chemotherapy efficacy, the antagonism or agonism of AHR may also play a role in immunotherapy. More specifically, recent progress has been made regarding inhibitors targeting Programmed Cell Death-1(PD-1) and programmed cell death ligand 1 (PD-L1). Conventionally speaking, PD1 biologically serves to prevent autoimmune diseases via downregulation and suppression of the immune system.[49] However, in the context of cancer, PD-1 is a leading factor contributing to immune system resistance within the tumor microenvironment.[49] A recent study had previously characterized the tumor repopulating cells (TRC) as a driver for PD1 upregulation in CTL's via the kynurenine (Kyn) and AHR transcellular pathway.[50] The association of AHR induction with PD-1 upregulation could likewise also be inhibited via AHR antagonism, promoting overall CTL apoptotic efficiency.[50]

In conclusion, our results further highlighted the complexity of the tumor microenvironment and the importance of context within cancer therapy. A novel antagonist IK10364 was tested and both TCDD and IK10364 were verified to induce and inhibit AHR activity respectively via *CYP1A1* relative mRNA expression levels. However, while AHR activity was confirmed, their respective phenotypes in consideration of HNSCC survival were contrary from our expectations. This leads to a possible explanation that both the AHR agonist and antagonist possess an ability to modify separate subset of genes enabling them to reach the same phenotype through different pathways. Additionally, cancer-related targets *LAT1, ABCG2, SESN2, CSF2, CSF3, IL6, IL1B,* and *MMP1* were all analyzed via qRT-PCR under TCDD induced AHR and vehicle conditions. Virtually all were involved in some form with tumorigenesis, and a statistically significant increase in mRNA expression level for the TCDD Induced AHR group relative to the control was found.

Additionally, limitations with the monolayer culture system were also discussed. As such, further study of AHR modulation should be done utilizing 3-D culture and spheroids serving as a better way to replicate the morphology, cell-cell, cell-ECM, nutrient deprived and other phenotypic conditions characteristic of a tumor. Likewise, an in-vivo experiment utilizing a mouse model should also be done to further characterize AHR modulation under context of cancer.

Appendix A

qRT-PCR

Table 1: Human qRT-PCR Primers

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
CYP1A1	TACCTCAGCAGCCACCTCCAAGAT	GAGGTCTTGAGGCCCTGAT
GAPDH	TGCACCAACTGTTTAGC	GGCATGGACTGTGGTCATGAG
B-ACTIN	CACCATTGGCAATGAGCGGTTC	AGCTCTTTGCGGATGTCCACGT
SLC7A5 (LAT1)	ACAGCTGTGAGGAGCAGCAC	TCTTCGCCACCTACTTGCTC
ABCG2	TTTCCAACGGTTCATTCAAAA	TACGACTGTGACAATGATCTGAGC
SESN2	AGATGGAGAGCCGCTTTGAGCT	CCGAGTGAAGTCCTCATATCCG
CSF2	GGAGCATGTGAATGCCATCCAG	CTGGAGGTCAAACATTTCTGAGAT
CSF3	CCAGAGCTTCCTGCTCAAGT	GTAGGTGGCACACTCACTCA
IL6	AAATTCGGTACATCCTCGACG	AGTGCCTCTTTGCTGCTTTCA
IL1B	TCTGTACCTGCTCGTCGTGTTGAA	TGCTTGAGAGGTGCTGATGTACCA
MMP1	AGTCCAGAAATACCTGGAAAAATA	TTTTTCAACCACTGGGCCGCCAC

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ACADEMIC VITA: Kebo Zhang

EDUCATION

Penn State University| Schreyer Honors College University Park, PA
Eberly College of Science: Biochemistry Molecular Biology Class of 2021

Awards

- Dean's List **09/2017 – 05/2021**
- Braddock Scholarship **09/2017 – 05/2021**

RESEARCH EXPERIENCE

Penn State Department of Veterinary Sciences **09/2018 – Present**
Undergraduate Research Assistant (Honors Thesis)

- Engaged in research regarding carcinogenesis under Dr. Perdew regarding the Aromatic Hydrocarbon Receptor.
- Identified receptor interactions with Head and Neck Carcinoma cell phenotype under agonism and antagonist conditions via RT-qPCR, Colony Forming Assay, and Cell Count Viability Assay.

Penn State Department of Chemistry **01/2018 – 05/2018**
Internship

- Conducted Research under Dr. Showalter concerning intrinsically ordered and disordered regions of PDX1.

Institute of Regenerative Medicine at University of Pennsylvania
Paid Internship and Research Fellowship **07/2017 – 08/2017**

- Selected one among 10 students out of a multitude of applicants throughout Pennsylvania to conduct research at the University of Pennsylvania Institute of Regenerative Medicine.
- Shadowed and supported Dr. Juxiang Yang (PhD) and Daphne Yau (MD) as they worked upon a collaborative project between the Stanescu Lab (University of Pennsylvania) and the Stanley Lab (Children's Hospital of Philadelphia).
- Analyzed and aided in the perfusion of Postnatal 3, 7, and 14 rats in the determination of glucokinase and hexokinase roles in insulin sensitivity throughout development.

HEALTHCARE EXPERIENCE

Rural Area Medical **09/2018 – 05/2021**

- Engaged in bringing free healthcare clinics and vaccination drives to Pennsylvania.
- Served at a Free Healthcare Clinic in Ashtabula Ohio.

Christian Medical Dental Association Chapter at Penn State (CMDA) **01/2020 – 05/2021**

- Founded a CMDA undergraduate chapter for students centered on integrating faith with professional STEM and Healthcare careers.

EXTRACURRICULAR EXPERIENCE

PSU Music Service Club: **09/2017 – 09/2019**

- Performed various concerts for retirement and nursing homes near State College

Serve State: **09/2017 – 09/2019**

- Participated in volunteering opportunities throughout the community (25 hour minimum per semester).

Asian American Christian Fellowship **08/2018 – 05/2021**

- Christian Community dedicated to spiritual emotional growth of members.

Organic Chemistry I and II **01/2019-05/2021**

- Aided Dr. Houck as a Chemistry Grader and Learning Assistant for Organic Chemistry