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Inflammation Of The Taste Sensory System: Cyclophosphamide And Amifostine

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INFLAMMATION OF THE TASTE SENSORY SYSTEM: CYCLOPHOSPHAMIDE AND AMIFOSTINE

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ABSTRACT

Chemotherapeutics are used extensively to treat cancer patients and often induce adverse effects, including taste dysfunctions. Disturbances in taste are detrimental to the overall well-being of cancer patients, causing malnutrition and weight loss that aggravate their condition even further. Inflammation due to an infection of the taste sensory system as previously shown, has detrimental effects on the taste sensation. Our study focused on if chemotherapy induced an inflammatory response in the taste buds using cyclophosphamide (CYP), a pro-drug. Once metabolized by the P450 enzyme complex, its primary metabolite functions as an alkylating agent, involved in inhibiting cell replication cycle and cell death. We used immunohistochemistry and fluorescent microscopy to analyze and observe the expression of the pro-inflammatory cytokine TNF-α in different types of taste sensory cells. Previously we found that a sulfhydryl cytoprotective drug, amifostine, can prevent taste bud damage and therefore we asked if it could prevent an inflammatory response. Our research observed an inflammatory response in both Type II and Type III cells in taste buds of fungiform and circumvallate papilla. Type II cells showed a peak response at 8- and 24-hour post-CYP injection whereas Type III cells had a peak expression at 24-hour post-CYP injection in the circumvallate papillae. Pre-treatment with amifostine appeared to prevent an inflammatory response within taste buds from CYP induced cytokine response. Identifying inflammation as a potential factor in taste related disorders could help clinicians develop better treatment modalities such as cytoprotective drugs preventing chemotherapy induced long term adverse effects such as malnutrition. In the future, we would like to expand our research to investigate expression of other pro-inflammatory cytokines and possible signaling mechanisms that could be responsible for CYP-induced inflammatory response in Type III cells.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

1.1. Overview

Taste is an important sensory modality found in animals for the main purpose of identifying food and nutrients before ingestion. In humans, the gustatory system has two main purposes, it helps to detect and differentiate between nutrients and harmful toxins. Also, signals from the peripheral gustatory system prepare the gut for proper digestion and absorption of nutrients by releasing digestive juices and enzymes. Alterations in taste are common side-effects in patients undergoing chemotherapy (Sánchez-Lara et al., 2010). Our study involves investigating alterations in the taste system due to cyclophosphamide (CYP), a chemotherapy drug with alkylating properties, in a mouse model to identify any inflammatory reaction in both fungiform and circumvallate papillae. We also studied Amifostine, a sulfhydryl drug, to see if it has any protective effects in ameliorating this inflammation. This chapter describes the significance of the taste sensory system and the relationship it has among multiple factors. Subsequent chapters describe the experimental results of this project in relation to previous research and the future direction of our experimental results.

1.2 Importance of taste

Like other major sensory systems such as olfaction, taste is an important sensory system which helps most non-human animals and humans perceive the world around us. The importance of taste stems from its function of protecting the gastrointestinal system against ingestion of harmful substances as well as help provide adequate nutrition to the individual. There are five different basic tastes that animals have been identified and characterized (sweet, bitter, sour, salty and umami) that perform specific functions (Chaudhari & Roper, 2010).

1.2.1 Sweet taste

Sweet taste originates from food containing carbohydrates. Foods judged as sweet are usually preferred by most organisms. Carbohydrates are naturally present in plants and are the most important source of energy for organisms. Even though infants do not have fully developed taste receptors, they readily ingest their mother's milk which is high in free sugars and glutamate. Therefore, our predilection towards sugary food could possibly begin there (Ramirez (1990). Other studies also indicate that carbohydrate is the main source of energy for a human diet. For example, it is widely known that brain function depends on a steady and quick source of glucose. Also, ingestion of carbohydrates increases glucose oxidation. Thus, carbohydrate laden food serves the purpose of providing a ready source of glucose (Jéquier, 1994).

1.2.2 Umami taste

The amino acid L-glutamate is the principal compound that elicits a unique taste called umami. Monosodium glutamate (MSG) is the prototypical example of umami, sometimes referred to as a "savory" taste. In 1908, glutamic acid was first extracted from seafood by a scientist named Ikeda. He determined that this compound gives a palatable savory sensation to food and categorized this as a basic taste called umami. MSG is used more frequently in the eastern part of the world than in the western nations (France Bellisle, 1999). Endogenous glutamate has numerous functions within the body. Being an amino acid, it is found in many peptides and proteins. Monosodium glutamate is one of the major amino acids present in food adding natural flavor. It is also an energy source in the body, involved in glutathione synthesis (Jinap & Hajeb, 2010). Food such as tomatoes and mushrooms have a distinct umami taste due to the presence of high quantities of glutamate.

A study conducted by a group in France found that MSG enhanced the palatability of food (F. Bellisle et al., 1991). While it can improve the palatability of foods, the addition of MSG in food has been a controversial issue, mainly by blaming glutamate for ill health. The phenomenon was even termed 'Chinese restaurant syndrome' (Kwok, 1968). Years of study have concluded and refuted any claims of ill-health due to MSG (Kenney, 1986).

1.2.3 Bitter taste

Bitter taste is an important taste modality as it is responsible for protecting the body against ingestion of harmful chemical substances. Bitter tasting compounds are not just aversive to humans, but to most other animal species as well, suggesting its importance in protecting against harm (Chandrashekar et al., 2000). This is quite evident in synthetic compounds or plant secondary metabolites that are generally harmful to the individual.

1.2.4 Salt taste

Salt taste is generally detected by the presence of sodium chloride and sodium chloride is implicated in a lot of chronic diseases in humans such as hypertension (Mattes, 1984). It is used as a seasoning in almost all foods to increase its palatability and we have an inherent need for sodium due to its life sustaining functions (Fitzsimons, 1998). It has been noted though that there is a difference in how rats and humans ingest sodium chloride. Humans like it with food and rats like it with fluid (Beauchamp & Bertino, 1985). Unlike sweet, humans have not been found to have an inherent predilection towards salty taste and are somewhat indifferent towards salt sensation. Their detection for salt taste stimuli develops over the first few months post birth, which is correlated with the development of central taste sensory structures (Beauchamp, Cowart, & Moran, 1986).

It has been noted that neonates do not specifically differentiate between salt and water which could be due to the lack of development of salt taste sensory system. This was corroborated by studies in sheep and rats exhibiting a lack of neural innervation and amiloride sensitive channels in taste cells. The taste sensation of salt also induces a wide array of physiologic changes which includes the secretion of saliva, gastric juices, and pancreatic enzymes that play key roles in metabolic processes within a human being (Hill & Mistretta, 1990).

1.2.5 Sour taste

Sour taste is an important sensory modality which denotes the presence of acid in food and is often used to determine whether a food is safe to eat or is rancid and should be discarded (Chaudhari & Roper, 2010). It is commonly found in nature in the form of acetic acid, tartaric acid or maleic acid in fruits and berries. Even though they are found ubiquitously in nature, the intensity and sensory characteristic of these acids differ widely (Törnwall et al., 2012). A sour substance is often used as an additive to enhance the flavor and the quality of the food to be ingested. These substances are also often used in baked goods, beverages and confections as an enhancer (Hartwig, 1995). Apart from this, acidulants are also used in pH control to prevent any microbial growth. Acidulants also help in gelling of food and sometimes used as a chelating agent (Johnson & Peterson, 1974). Early studies based on inorganic salts suggested that hydrogen ions were responsible for sour taste sensation. Later studies found that the sour taste elicited by organic acids was due to hydrogen ions dissociating from carboxyl groups. Studies performed to investigate the differences in intensity of different organic and inorganic acids based on molar

concentrations, suggest the intensity of sour sensation elicited by acids is not solely depend on molar concentrations (Makhlouf & Blum, 1972).

1.3. General morphology of the taste buds

The taste sensory organ or taste bud is an onion-shaped structure comprised of about 50~100 differentiated taste cells, referred to as Types I, II, and III, in the taste papillae on the tongue. These three differentiated taste cells types are responsible for the transduction of five basic tastes: sweet, bitter, umami, sour and salty. Taste buds are organized within papillae called fungiform, foliate and circumvallate. Fungiform papillae are found on the anterior end of tongue. Foliate are located on the lateral sides of the tongue and circumvallate are found in the center posterior part of the tongue. Taste cells form tight junctions preventing any permeation of water but allow ions such as Na+ to permeate into the interstitial spaces, which is one of the ways taste cells detect Na+ (Chaudhari & Roper, 2010).

Taste cells are differentiated polarized cells that have short life spans. They are known to undergo renewal due to their closeness to the external environment using the progenitor K14+/K5+ cells (Chandrashekar, Hoon, Ryba, & Zuker, 2006; Okubo, Clark, & Hogan, 2009). These progenitor cells are in the basal epithelial layer, immediately below the onion shaped taste buds. Post mitotic cells differentiate to form mature TSCs when proper signals are induced from the taste bud (Adler et al., 2000; Barlow, 2015). The apical ends of the mature differentiated taste sensory cell encounter a nutrient through the pore of the taste bud. When a food molecule encounters receptors in the microvilli of taste sensory cells, a transduction pathway is activated by the food molecule. Electron micrograph analysis of different taste buds have shown discrete ultrastructural features such as

differing electron densities of cytoplasm, thickness of the microvilli, difference in the kind of receptors on cells (for example, the presence or absence of synapses in cells) or the difference in thickness of microvilli in cells (Pumplin, Yu, & Smith, 1997). Antibody labeling of cells identified protein markers either involved in downstream signaling pathways, receptors or cell structure have been developed to help distinguish individual cell types from each other. For example, alpha gustducin is involved in the downstream cell signaling in Type II cells and SNAP25 is a core complex of the SNARE involved in exocytosis of neurotransmitter vesicles in Type III cells (Yang, Crowley, Rock, & Kinnamon, 2000). Understanding the differences between the mature cell types is important for studying the taste system.

1.3.1 Type I taste cells

Type I cells are among the most ubiquitous cells in taste buds and are involved in salt taste transduction. These cells are spindle shaped, extending all the way from the taste bud's basement membrane, with its microvilli projecting from its apical ends close to the taste bud pore. The cytoplasm of these cells does not have many organelles except for a distinctive Golgi-apparatus and dense bodies. Based on the cytoplasmic staining properties due to basophilic dyes, and varying electron density, these cells have been called dark cells. These cells are known to form tight junctions and the lamellate processes surround the neighboring cells within the taste bud like a glial cell (Farbman, Albert I. 1965). These cells are widely found within the taste bud with a population equivalent to 40% of the entire population (Finger, 2005). They have glial-like functions in taste buds, expressing different receptors such as GLAST or NTPDase2. The presence of GLAST indicates it is involved in glutamate uptake and the NTPDase2 nucleosidase hydrolyzes extracellular ATP, a

neurotransmitter in taste cells. Thereby, clearing excess neurotransmitter around the cells (Bartel, Sullivan, Lavoie, Sévigny, & Finger, 2006; Finger et al., 2005; Lawton, Furness, Lindemann, & Hackney, 2000). It also expresses the potassium channel, ROMK, involved in K+ ion homeostasis in cells. Type I cells eliminate K+ ions from the interstitial spaces of cells (Dvoryanchikov, Sinclair, Perea-Martinez, Wang, & Chaudhari, 2009).

1.3.2 Type II taste cells

Type II cells are elongated taste sensory cells, that extend from the taste pore to the base of the taste bud. They have a large nucleus and have transmembrane G protein coupled receptors (GPCR) in the plasma membrane for transduction of sweet, bitter and umami taste (Tomchik, Berg, Kim, Chaudhari, & Roper, 2007). These cells are characterized by an abundance of mitochondria and vacuoles spread across the cytoplasm (Farbman, 1965).

It has been hypothesized that these cells play a role in the maintenance of taste cells. Once GPCRs are activated by their specific ligands (taste stimuli), it transduces the downstream signaling pathway by a specialized G-alpha subunit specific for a taste stimulus (Nelson et al., 2001). This activates a second messenger pathway that leads to the release of a neurotransmitter. Type II cells are characterized by the afferent nerves close to the cell instead of forming pre-synaptic endings (R. G Murray, 1969; Ruibiao, 2000). The signal is transmitted to the nerve endings by the release of ATP functioning as a neurotransmitter, induced by the voltage gated Na+ and K+ ion channels present within the cell membranes. The ATP neurotransmitter release is through connexin or pannexin hemichannels (Nelson et al., 2001) where it can activate either Type III cells or the adjacent nerve fiber through P2X2/P2X3 receptors.

1.3.3 Type III taste sensory cells

Type III cells are very similar to the Type II cells in their shape and electron density but characterized by its abundant presence of numerous endoplasmic reticula. Type III cells are characterized by pre-synaptic nerve endings that innervate the taste sensory cell membrane. These cells are also characterized by the presence of ubiquitin carboxyl terminal hydrolase PGP9.5, Serotonin 5-HT and neuron specific enolase, commonly found in rats and mice. These cells are known to be found more centrally than peripherally within the taste bud with a single microvillus at its apical end (Yee, Yang, Böttger, Finger, & Kinnamon, 2001).

These cells are known to express proteins such as SNARE complexes, involved in synaptic junctions with nerve terminals & Fujimoto, 1969; Ruibiao et al., 2000). Neuronal-like genes expressed by these cells produce NCAM, enzymes to synthesize neurotransmitters, an adhesion molecule over the cell surface, and voltage-gated Ca2+ channels for neurotransmitter release. These are presynaptic cells that express a range of voltage-gated Na and K channels to induce action potentials. These cells are also known to respond to signals generated by nearby cells (DeFazio et al., 2006; Gao et al., 2009; Medler, Margolskee, & Kinnamon, 2003; Vandenbeuch & Kinnamon, 2009). The likely neurotransmitter for these cells is serotonin.

1.3.4 Basal cells

Basal cells are often identified as Type IV cells (Barlow, 2015). These are immature, post-mitotic cells, located at the base of the taste bud. Transit amplifying cells divide symmetrically to give rise to the basal cells. These migrate into the taste bud under the

influence of proper signaling mechanism within the taste bud to differentiate into mature taste sensory cells, such as Type I, II or III.

1.4 TRANSDUCTION OF TASTE STIMULI

1.4.1 Salt taste transduction.

The primary salt taste transducer has been identified to be amiloride-sensitive epithelial sodium channels (ENaCs) which are widely present in the Type I taste sensory cells. These channels allow the influx of sodium ions into the taste sensory cells to depolarize it. These ion channels are known to be inhibited by amiloride in micromolar quantities. ENaC channels are composed of three homologous α-, β- and γ-ENaC subunits. All three subunits of ENaC channels are found in all papillae present in the tongue but at different degrees (Gilbertson, Damak, & Margolskee, 2000). *In vitro* preparation of rat lingual epithelial cells, when exposed to sodium chloride, induces a slow inward current which is reduced when amiloride is introduced (Heck, Mierson, & DeSimone, 1984). Influx of Na+ ions induce a voltage-gated outward current.

Recent studies with Types II and III cells have also provided some indication that they could certainly be involved in salt taste transduction. Calmodulin (CALHM1) a group of hexamer channels present in the cell membrane of Type II cells involved in ATP release, could be implicated in salt taste transduction. Experiments conducted using CALMH1 knockout animals showed smaller responses to NaCl stimuli in the chorda tympani nerve of knockout animal than Wild Type animals (Tordoff et al., 2014).

Amiloride-insensitive salt responses were exhibited by Type III cells which responded to the anions instead of cations to induce a transepithelial potential. Single cell

calcium imaging showed responses to anions in these sour taste sensing cells (Lewandowski, Sukumaran, Margolskee, & Bachmanov, 2016).

1.4.2 Transduction of Sweet, Bitter and Umami

Type II taste sensory cells are responsible for sweet, bitter and umami taste transduction. These taste stimuli are detected by a heterogenous population of receptors activating transmembrane GPCRs to induce the downstream signaling pathway for the taste stimulus. The family of GPCRs involved in taste transduction in Type II cells comprises of three distinct groups: mGluR, T1Rs, and T2Rs (Chandrashekar et al., 2000a; Jiang et al., 2004; Simon, de Araujo, Gutierrez, & Nicolelis, 2006)

The T2R family of receptors are involved in bitter taste detection and transduction. Different sets of genes encode T2R receptors in the taste cells (Chandrashekar et al., 2000b). These range between 20-35 different genes among mammals. These taste receptors have varying degrees of receptivity towards taste stimuli. Some of these receptors are either narrowly or broadly tuned, which means some of the receptors identify and transduce a specific set of ligands, whereas others identify a range of different ligands (Meyerhof et al., 2010). In-situ hybridization has shown T2R mRNAs are co-expressed in the receptor cells. Partially overlapping nature of taste receptors explains our ability to discriminate between different bitter taste stimuli.

The T1R receptor family is comprised of three receptors that form heterodimeric pairs. The heterodimeric form of T1R receptors, T1R2+T1R3, is responsible for the detection of sugars and artificial sweeteners. Despite of lacking T1R3, some mice are still able to detect sweet taste stimuli, indicating other, unidentified receptors are also involved in detection of sweet substances (Damak et al., 2003). A2B adenosine receptors has been

implicated in the enhancement of sweet taste reception. Prior research has shown the ATP released from the cells were broken down to adenosine which evoked Ca2+ signaling in cells to enhance the sweet taste in the presence of stimuli (Dando, Dvoryanchikov, Pereira, Chaudhari, & Roper, 2012).

Previous research showed heterodimeric receptors T1R1+T1R3 are involved in umami taste stimuli. Umami taste is characterized by L-glutamate and GMP/IMP compounds. These compounds are indicators of protein in food. Further research using ratiometric calcium imaging with Fura-2 of isolated taste sensory cells from C57BL/6J and T1R3 knockout animals showed physiological responses when stimulated with L-glutamate, L-serine and IMP. These data suggest that these cells responded to a broad array of amino acids and that there are additional receptors involved in umami taste detection. Recent behavioral and calcium imaging experiments have shown that mGluR receptors also respond to glutamate and other L-amino acids (Choudhuri, Delay, & Delay, 2015)

T1R family of receptors are categorized within the class III GPCRs, with N-terminal extracellular domains forming a Venus flytrap structure for ligand binding (Cui et al., 2006; Max et al., 2001). T2Rs are categorized as a class I GPCR with transmembrane binding sites (Floriano et al., 2006). Binding of the ligand to members of either family of 7 transmembrane GPCRs induces conformational changes that activates heterotrimeric G-proteins. The heterotrimeric G-proteins are composed of alpha, beta and gamma subunits. Activation of the G-protein is characterized by the coupling of GTP to the alpha subunit and the separation of the alpha subunit from the beta-gamma subunit (Spielman, 1998). Furthermore, with the activation of the receptor, the βγ is released to induce the downstream signaling pathway by interacting with a phospholipase C (PLCβ2). The

phospholipase stimulates IP3 production to open the endoplasmic reticulum to release Ca2+ ions into the cytosol. The released Ca2+ ions target both TRPM5 channels responsible for Na+ ion influx and a gap junction hemichannel, responsible for ATP neurotransmitter release (Simon et al., 2006)

1.4.3 Sour taste transduction

Sour taste is a result of acid concentrations within the vicinity of taste sensory cells in the tongue. The concentration of H+ ions determines the sourness of the stimulus. Like other tastes, sour taste also seems to be modulated by specialized receptors at the periphery. Studies suggest the polycystic kidney disease-like ion channel (PKD2L1) may play a role in sour detection. One of these studies used bioinformatics screening and in-situ hybridization technique to identify the receptor's involvement in sour taste transduction. This study involved genetic ablation of taste cells with diphtheria toxin to induce loss of specific taste receptors to see if there was a loss of sour taste. Ablated T1R resulted in the loss of only sweet taste. Similarly, loss of PKD2L1 saw a loss of acid taste (Huang et al., 2006). In-situ hybridization and double-labeled fluorescent immunohistochemistry was conducted to investigate the co-expression of receptors specific for different tastes such as T1R, T2R or TrpM5 with the candidate sour taste receptor, PKD2L1. The result showed no co-expression of the mentioned receptors with PKD2L1.

1.5 TASTE BUD DEVELOPMENT AND RENEWAL

1.5.1 Brief timeline of taste bud formation

The development of taste buds has been investigated in mice beginning at an embryonic stage until clear differentiation of the taste structure occurs after birth. At an

embryonic stage day E11, a rudimentary structure of tongue forms, characterized mostly by its epithelial bilayer. At embryonic stage E12.5 taste placode formation occurs, which is basically the arrangement of undifferentiated columnar cells beneath the epithelial bilayer, which eventually forms the differentiated taste cells. The placodes are surrounded by sensory nerves. At E15.5-18.5 taste papillae morphogenesis begins with the formation of the mushroom shaped structure, an apical localization of the immature taste buds and sensory nerve innervation. About a week after birth, the mature taste bud appears in fungiform and circumvallate papillary structures (Barlow, 2015; Kapsimali & Barlow, 2013).

1.5.2 Taste bud cell renewal

Taste cells have an average life span of about 10-14 days in rodents, suggesting it gets continually renewed within a short span of time (Beidler & Smallman, 1965; Farbman, 1980). During any given day, about 10% of cells are lost due to natural attrition, 20-30% of the cells are new and differentiating simultaneously, the remaining 60-70% of the cells are functional taste cells (Barlow, 2015).

Studies using inducible lineage tracing techniques have identified cytokeratin, K5 and K14 markers, in the taste progenitor cells. These progenitor cells are responsible for replacing the cells within the taste buds. These progenitor cells self-renew and divide to give rise to the ovoid-shaped basal cells. The basal cells or Type IV cells reside within the basal region of taste buds, migrating to the taste bud structure under proper signals from within the bud. The basal cells express sonic hedgehog (Shh) signaling to regulate the cell renewal process (Miura, Scott, Harada, & Barlow, 2014). Subsequently, the immature

basal cells migrate into and mature within the taste bud structure to give rise to the different types of taste cells.

1.5.3 Signaling mechanism for taste bud development and renewal

1.5.3.1 Hedgehog Signaling. Shh is a factor generally expressed by the taste bud basal cells. Shh induces transcription of its target genes Gli1 and Ptch1 in progenitor cells located outside the taste buds and are involved in regulating cell replacement in taste buds. Shh's role as a cell regulating factor was revealed by developmental experiments where inducible genetic model expression was used. This study also showed that excess Shh induces an overexpression of ectopic taste buds on the anterior filiform section of the tongue (Castillo et al., 2014). Drug-induced disruptions in the Shh signaling pathway have resulted in taste-related complaints from patients undergoing chemotherapy. Some of these drugs directly impact factors such as Smoothened (Smo) within the Hedgehog signaling pathway, thereby disrupting the normal cell differentiation cycle resulting in a reduced number of taste cells over a period and a loss of taste function. But this loss in taste is reversible and comes back with the removal of the drug (Kumari et al., 2015).

1.5.3.2 Wnt/β-catenin Signaling. Embryonic taste bud or taste placode development is induced by the Wnt/β-catenin pathway within the taste bud. The Wnt pathway remains active in the adult mature taste cells, specifically the BATGAL reporter mice expressing β-galactosidase in the presence of β-catenin. Its presence is detected all the way from the progenitor cells until the mature taste sensory cells, implicating its importance in cell lineage within the taste system (Gaillard & Barlow, 2011).

This signaling pathway also plays an important role in the mature taste bud. For example, BATGAL reporter mice express beta-galactosidase in progenitor cells as well

as in taste sensory cells in the presence of b-catenin, suggesting this pathway is responsible in taste cell renewal to give rise to the mature taste sensory cells. The role of β-catenin was tested by overexpressing β-catenin in K5+ basal keratinocytes and observing differentiation of K5+ cells into mature taste sensory cells, predominantly Type I cells (Barlow, 2015).

1.6 GUSTATORY AFFERENT TASTE PATHWAY

Initially information about taste begins with taste receptors. This information is transmitted by the cranial nerves located close to the anterior and posterior end of the tongue to the brain stem. Each taste bud in humans is innervated by 4-14 sensory neurons which penetrate the basal lamina to enter the taste buds and are extensions of cranial nerves. The anterior 2/3rd of the tongue is penetrated by cranial nerve VII, the facial nerve, and the posterior end of the tongue is innervated by the glossopharyngeal nerve, cranial nerve IX. Finally, the third set of cranial nerves is known as the vagus nerve (cranial nerve X) surrounding the throat and its adjacent area (Hamilton & Norgren, 1984). The cell bodies of the neurons are present in geniculate, petrosal and nodose ganglia of cranial nerves VII, IX, and X, respectively. Gustatory nerve fibers co-mingle as a plexus in the oral cavity under the taste epithelium (Krimm & Hill 1998; Vandenbeuch & Kinnamon, 2009). It is almost impossible to determine the nerve fibers within a taste bud responsible for specific taste information due to the unavailability of clear marker of those neurons to distinguish between different types of neurons (Farbman, 1965). Immunostaining and retrograde tracing have helped identify the taste pathway in rats from the peripheral region to the sensory cortex. Neurons link each structure of the pathway. It begins at the nodose ganglion

(periphery) which sends axons to the solitary nucleus in the medulla. Projection cells in this nucleus send axons all the way up to parabrachial nucleus of the pons. These neurons project their axons to the ventral posterior medial nucleus in the thalamus which subsequently send their axons to the taste sensory cortex of the rodent brain (Hamilton & Norgren, 1984).

1.7 TASTE CODING

Taste coding is referred to as the process by which the peripheral signal detected by the taste sensory cells is converted to neural signals for the transmission to the brain cortical region. There currently are two theories of taste information coding. Labelled line model states that a basic taste is transduced by only one type of taste receptor and transmitted by one type of nerve fiber associated with that taste. The across neuron pattern model states that a taste can be transduced by different receptors and the information is transmitted to the brain by more than one type of nerve fiber (Erickson, 1982; Hellekant, Ninomiya, & Danilova, 1998) and taste identification is determined by the collective neural codes of all fibers conveying the information.

Evidence towards labeled line coding can be found in T2R receptor isolation, G-protein coupled receptors, which are expressed in subsets of taste sensory cells found in the tongue and palate. T2Rs are responsible for detecting bitter taste stimuli exclusively, even though both humans and mice cannot differentiate among different forms of bitter compounds. The study used heterologous expression system to identify the receptors' function in detecting bitter stimulus. Both the human and the mouse T2R receptors responded to denatonium and cycloheximide respectively (Adler et al., 2000;

Chandrashekar et al., 2000). T2Rs discriminating different types of bitter taste is an indication of broader coding in cells. On the contrary, these cells are exclusively expressed in α-gustducin expressing cells, also known as the Type II cells is an indication of labeled line coding.

Labeled line coding in taste could also be seen in sweet taste transduction, which is detected by T1R family of receptors. Sweet is detected and transduced by heterodimeric combination of T1R2 and T1R3 receptors. It can detect a wide range of sweet tasting compounds such as sucrose, saccharin or dulcin (Nelson et al., 2001). Even though its exclusive detection of sweet taste suggests a labeled line coding towards a specific taste response, its inability to differentiate among different sweet tasting compounds suggest an across fiber pattern coding within the periphery (Ahmed & Hombal, 1984).

With regards to the taste coding within the cortical regions of the brain some studies suggest the presence of specific regions or a group of neurons which exclusively respond to specific taste stimuli. For example, a study conducted on mice using *in-vivo* two photon calcium imaging suggests different topographic segregation within the cortical regions (Zhang et al., 2003).

Across fiber pattern coding in the cortical region could also be understood and best represented by the relative levels of activity in the afferent neuron population carrying information to the cortical region. A study involving whole mouth stimulation with various compounds have shown the presence of a groups of neurons responding to a stimulus better than others, even though responding to other stimuli as well. Utilization of bitter compounds have detected the presence of bitter best neurons responding mostly to a variety of bitter stimuli (Geran & Travers, 2006).

All the above studies suggest a combination of different types of coding processes within the taste sensory system. These processes begin at the periphery and end up within the cortical region of the brain to process the taste information.

1.8 CHEMOTHERAPY DRUGS

Cancer is a disease affecting millions worldwide. The predominant mode of treatment is either chemotherapy or radiation or their combination. Chemotherapy involves the use of toxic drugs to disrupt the cell replication cycle by inducing cell death of proliferating cancer cells. Chemotherapy drugs interact with rapidly dividing cells and are non-specific in action to induce cell death. Use of these drugs therefore results in cell death at various regions of the body where normal cells are proliferating, hence its side effects. Chemotherapy drugs are classified into different classes, based on its mode of action. They are the following: alkylating agents, antimetabolites and topoisomerase inhibitors.

1.8.1 Alkylating Agents

These drugs are among the first developed drugs used for cancer treatment. They were developed during the World War I era (Corrie, 2008). These drugs can alkylate DNA or RNA by transferring an alkyl group to the strand via covalent bonds. Subsequent replication cycle results in strand breaks (Lind, 2008). These drugs non-selectively target proliferating tumor and normal cells to induce cell death (Corrie, 2008). Due to their non-selective nature, these drugs have a dose-dependent toxic effect over the cells. A few different subtypes of this type of drugs are nitrosourea, nitrogen mustards, tetra-zines, cisplatin and cyclophosphamide (Giorgi-Renault et al., 1988).

1.8.2 Antimetabolites

Anti-metabolites are a group of drugs or molecules that prevent the synthesis of DNA or RNA. Instead of the usual nucleotides synthesizing into long chains of DNA, it is replaced by chemical groups similar in structure to the nucleotides or nucleosides. They also induce its effect by inhibiting the enzymes involved in the normal synthesis of DNA (Parker, 2009). For example, anti-metabolites such as methotrexate inhibits dihydrofolate reductase (DHFR). This enzyme is involved in purine production, essential for DNA synthesis (Hillcoat, Swett, & Bertino, 1967).

1.8.3 Topoisomerase inhibitors

Topoisomerase inhibitors are chemical compounds affecting the normal activity of topoisomerase I and II. The torsion created due to the processes of replication and transcription cause an overwound DNA double strand, thus not allowing DNA polymerase to traverse down the double stranded DNA. Hence, topoisomerases are enzymes responsible for creating temporary nicks within the DNA double or single strand to release the stress induced by these processes. The topoisomerases are classified into sub-types I and II (Champoux, 2001). Topoisomerase I inhibitors are involved in trapping the intermediate complex of TOPI cleavage complex, rendering it useless. Camptothecin-derived compounds belong to this category (Pommier, 2006).

1.8.4 Cyclophosphamide

Cyclophosphamide is one of the earliest and most effective anti-neoplastic drugs formulated and is still in use. Cyclophosphamide is an anti-neoplastic drug belonging to the class called alkylating agents and has immunosuppressive properties. It is used in the treatment of different forms of cancer including leukemia, lymphoma and breast cancer.

This drug kills rapidly proliferating cells and resting lymphoid cells. It is also regularly used post tissue transplantation and diseases which could be a result of inflammation (Gershwin et al, 1974; Orjuela et al., 2003). Alopecia, nausea, myelosuppression or hemorrhagic cystitis are just a few of the toxic effects (Ahmed & Hombal, 1984). Even though the drug is predominantly used for the treatment of cancer, it can also induce cancer in patients. Cyclophosphamide is also used in the treatment of neuroblastoma and carcinomas of different organ system such as lung, breast and gastrointestinal systems (Gershwin et al., 1974)

1.8.4.1 Metabolism. Metabolism of this compound occurs within the liver, induced by the cytochrome P450 isoforms, CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18, and 2C19. The two metabolites formed are phosphoramide mustard and acrolein. Phosphoramide mustard is the compound responsible for most of its antitumor or the chemotherapeutic effects. Acrolein has been implicated in a wide range of diseases including multiple sclerosis, cardiovascular diseases among others (Ramu, et al. 1995; Moghe et al., 2015). In cells it can cause mitochondrial damage or oxidative stress (Moghe et al., 2015). Metabolic pathway for the drug begins with activation within the hepatic cells. The cytochrome P450 enzyme complex oxidizes the compound to its active metabolites 4-hydroxycylophosphamide and aldophosphamide which exists in its tautomeric form. It is then further oxidized to carboxyphosphamide. The aldophosphamide diffuses into the cells to metabolize into the active compound phosphoramide-mustard and acrolein (McDonald et al., 2003).

1.8.4.2 Mechanism of Action. The active metabolites from the drug, phosphoramide mustard and to some extent acrolein, are known to be involved in anti-cancer activity.

Phosphoramide mustard targets the N7 guanine ring of the DNA strands cross-linking the DNA in proliferating cells to disrupt the cell cycle and, thus, prevents replication of cells. Even though phosphoramide mustard is known to cause off target damage to normal cells, the drug still exhibits quite a lot of specificity as the metabolite is hydrophilic and studies show the tumor cell walls to be hydrophilic (Brock & Hohorst, 1967; Fleming, 1997).

1.8.5 Effect of chemotherapy on taste

Chemotherapy has a variety of side effects which includes the loss of immunity to infections and the loss of sensitivity to taste and smell stimulation. Disruptions in taste and smell can have a significant effect on the overall health and quality of the individual, both psychologically and physically. The inability to taste food can result in a reduced intake of nutritious food, thwarting the recovery of the patient and thus leading to a poor psychological health (Comeau et al., 2001). Studies suggest that after chemotherapy treatment, at least 40% patients are malnourished. Malnourishment results in a significant decrease in the response to therapy (Comeau et al., 2001). Chemotherapy drugs that are generally associated with taste-related dysfunctions are cisplatin, carboplatin or cyclophosphamide to name just a few. Several studies with patients undergoing chemotherapy have been conducted to identify the effect of these drugs on taste. One study involving a small group of patients helped document the kind of taste changes that occur with chemotherapy. Participants described tastes as either metallic, stale or musty. Different patients described different taste dysfunctions. Some could detect sweet food and others would dislike it or could not detect sweet at all. Similarly, some patients would have a distorted sense of taste and would, for example, describe the taste of coffee as unusually burnt (Bernhardson, Tishelman, & Rutqvist, 2007). Another study was conducted on a total

of 518 patients, of which at least 134 patients developed taste-related changes. Some patients had an immediate effect of drug treatment on their taste whereas others seem to have an effect 10 weeks post first treatment. Most patients reported dysfunction in sweet and salty taste sensation (Bernhardson, Tishelman, & Rutqvist, 2008).

Based on these patient accounts of taste-related changes, behavioral, cellular and molecular studies have worked extensively using a mouse model to identify the underlying cause of these changes. One study examining the effects of cyclophosphamide on umami taste, found a two-phased drop in taste sensitivity after injection of 75mg/kg of the drug. The first drop was associated with a sharp drop in the number of fungiform papillae which did not show improvement in number until 12 days post-injection. A similar decrease in the number of taste buds for circumvallate papillae began about 8 days post injection (Mukherjee & Delay, 2011). Morphological studies like these, demonstrate the decline in taste papillae has a significant role for drug-related changes in taste. A more immediate effect of the drug on taste sensory cells was observed in a study using cell death assays to examine the effects of cyclophosphamide. TUNEL assays on fungiform and circumvallate taste buds show a peak in cell death at around 6-8 hours post injection. Caspase-3 assay of apoptosis showed that a second wave of cell death began 12 hours post injection, peaked at 18-24 hours and returned to control levels within 48 hours. This suggests that the drug acts quite quickly resulting in taste cell loss from within the taste papillae (Mukherjee et al., 2017).

1.9 CYTOPROTECTIVE AGENTS

Chemotherapy or radiotherapy commonly used to treat cancer, have their own side effects and toxicities, which could be acute, for a short phase or it could be cumulative,

leading to severe consequences, sometimes death (Rose, 1996). Often to mitigate these effects, the dosage for the drugs or radiation is modulated within its therapeutic index, which is the ratio of the therapeutic effect of the drug to the toxicity caused by it. There are no fixed and effective treatment procedure that could be involved to prevent toxicity related to these drugs but use of cytoprotective agents such as amifostine could help reduce its harmful effect. One very important aspect of these protective agents is their ability to preferentially treat normal cells from cancerous or damaged cells. Amifostine has been extensively studied and its efficacy in preventing damage induced by chemotherapeutic has been established (Mukherjee et al., 2013)

1.9.1 Amifostine

Amifostine is a thiol-based sulfhydryl drug developed by Walter Reed Army institute of Research. About 4000 sulfhydryl compounds were tested for their cytoprotective effects, out of which only one compound, WR-2721 also known as amifostine, was chosen (Grdina, Kataoka, & Murley, 2000). Amifostine is one of the few drugs providing protection to normal cells from chemotherapy-induced damage (Yuhas, 1980). This drug has been approved by FDA for both chemotherapy as well as radiotherapy and it has been extensively used to prevent xerostomia in patients undergoing head and neck radiotherapy (Rose, 1996).

1.9.2 Pharmacology of amifostine

Amifostine is a hydrophilic compound and, therefore, does not generally cross cell membranes. It is a pro-drug, which means that it remains inactive in its native state and is activated only after it dephosphorylates to its active metabolite WR1065 either due to the hydrolysis catalyzed by alkaline phosphatase in normal cells or due to spontaneous

hydrolysis within cells. This compound's therapeutic property is determined by greater uptake of this compound in normal tissues than in tumor tissues (Yuhas, 1980; Utley et al., 1984). This differential uptake of the drug can be further explained by the fact that an acidic environment within tumor cells prevents the activity of alkaline phosphatase within cells as well as the abnormal vasculature of tumor tissues (Calabro-Jones, Fahey, Smoluk, & Ward, 1985). The active metabolite of the drug is eliminated having a half-life of about 30 minutes in lung and skin, whereas it is eliminated more slowly in salivary glands which requires about 3 hours (Utley et al., 1984).

1.9.3 Mechanism of Action

The mode of action of amifostine begins with the metabolism of the drug to its individual active metabolites WR1065 and WR33278. It is believed that both compounds are involved in scavenging free radicals within the cells in competition with oxygen. At an intermediate level of oxygen, the protection is maximum. High oxygen levels result in the loss of protection whereas at low cellular oxygen level, the metabolites do not provide any protection because the cells are self-protected. In addition, the metabolite WR33278 is structurally like naturally occurring polyamines which are involved in DNA stabilization and repair, and therefore has an antimutagenic effect in cells (Perrin, Kataoka, Grdina, & Basic, 1992).

1.9.4 Toxicity and administration of the drug

Amifostine does not appear to have a major toxic effect but it does have some dose-dependent effects that require attention. A higher dose than what should be administered, could potentially result in hypotension. When used with radiotherapy, a patient requires close monitoring at lower doses due to the possibility of hypotensive conditions.

Antiemetics are generally administered along with the therapy to prevent nausea and vomiting, which is a possibility at higher doses (Andreassen, Grau, & Lindegaard, 2003).

Phase 2 trials of the drug suggested an acceptable dose of 740 to 910 mg/m^2 as a cytoprotective agent. Furthermore, FDA suggested a dose of 910 mg/m^2 pre-injection in patients undergoing cisplatin-based chemotherapy to prevent nephrotoxicity in patients whereas a recommended dose of 200 mg/m^2 is to prevent radiation-induced xerostomia. Most preferable mode of administration has been via a subcutaneous injection. Both phase 1 and phase 2 trials using subcutaneous injection, have suggested very promising results. Benefits have been lower incidents of hypotension, bladder toxicity and mucosal reactions in patients (Andreassen et al., 2003;Koukourakis et al., 2000).

1.10 INFLAMMATION

Inflammation is an immune response to drug or infection induced injury to cells and tissues. Inflammation is characterized by redness, swelling or vascular permeability within tissues resulting in the accumulation of immune cells and small molecule cytokines, involved in a cellular or humoral immune response to pathogens at the site of injury. There are different soluble factors involved in immune responses which can be categorized into groups such as: 1) lipid metabolites, platelet activating factor (PAF) or prostaglandins, 2) soluble proteases or substrates releasing multiple pro-inflammatory peptides, 3) compounds such as nitric oxide, which is a vasodilator, and 4) small protein molecules released by immune cells called cytokines which could have both a negative or a positive effect over cells and tissues. For our study, the focus will be on cytokines as an inflammatory mediator (Feghali & Wright, 1997). For the next few paragraphs my

discussion will be on cytokines commonly found within the gustatory papillae. Some of the cytokines involved in acute inflammation are the interleukins IL-1, IL-6, IL-8 or Tumor Necrosis Factor-α (TNFα). Inflammation is a component due to which an immune response within the cellular system is initiated, possibly leading to a rapid cell death. Thus, this section discusses the introduces inflammation and its components such as the cytokines.

1.10.1 Interleukin-1

IL-1 α and β are encoded by different genes located in the chromosome 2 and are of the size 22-31kDa and 17.5kDa. These molecules are secreted by different types of immune cells such as phagocytes, fibroblasts or lymphocytes. These molecules are known to trigger inflammation in the form of fever by inducing the production of prostaglandins E2 (PGE2) by the vascular endothelium and the release of histamines from mast cells. Histamines are known to cause vasodilation within blood vessels. The pro-inflammatory effect of the cytokine IL-1 can be inhibited by IL-1 receptor antagonist such as IL-1ra, a naturally occurring inhibitor having physiological functions in the humans (Feghali & Wright, 1997; Warren, 1990; Dinarello et al., 1991).

1.10.2 Tumor Necrosis Factor

Tumor necrosis factor is found both as an α and β form. They are 17kDa and 25kDa in weight. TNF-α exists as a trimer and is produced by macrophages, fibroblasts, mast cells and NK cells. This is a pro-inflammatory cytokine which shares inflammatory properties with IL-1 such as the production of prostaglandins. TNF-α is also known to induce the production of other cytokines such as IL-6 in several other cell types. During infection from bacteria, induced by lipopolysaccharides, macrophages release TNF-α and its effects

are both autocrine, paracrine and endocrine. TNF is known to cause major metabolic disruptions and well as associated with some cancers (Idriss & Naismith, 2000).

1.10.3 Receptors for Cytokines

Cytokines elicit their responses in cells by binding to cell surface receptors to induce downstream signaling pathways. For example, the IL-6R comprises of two subunits, an 80kDa ligand binding molecule and another 130kDa signal transducing subunit. Tumor necrosis factor receptors are 55 and 75kDa in size that binds both TNF-α and β (Idriss et al., 2000). Both receptors share some identity in its extracellular domain. Even though cytokines bind to specific receptors, some share common signaling pathways inducing a cellular response. For example, IL-6 and IL-11 even after having different receptors share a common downstream signaling pathways (Feghali & Wright, 1997).

1.10.4 Inflammation and its implication on taste disorders

Extensive research on inflammation within the taste sensory organ has shown the presence of inflammatory cytokines and signaling mechanism in the taste sensory cells. Findings reveal the presence of TLR receptors and interferon signaling pathways in different types of taste sensory cells. As earlier mentioned, the inflammatory pathways and cytokines are essential for maintaining the integrity of taste buds but could be severely deleterious if expressed at a higher level (Wang, Zhou, Brand, & Huang, 2009).

Most of the studies on inflammation in the taste sensory system have been to investigate an infection induced inflammatory model either by using Lipopolysaccharide or RNA polycytidylic acid. Wang's studies have corroborated, for example, the presence of cytokines such as IL-10 an anti-inflammatory cytokine responsible in maintaining the integrity of the taste buds, as well as TNF-α, a pro-inflammatory cytokine important in cell

growth and survival in specific subsets of taste sensory cells, primarily in the Type II cells. It must be noted the presence of both pro- and anti-inflammatory cytokines suggest a physiological phenomenon to compensate for the effect of either of the cytokines (Feng et al., 2014; Feng, Zhao, Chai, Huang, & Wang, 2012).

Similar work to identify an Interferon signaling pathway and its receptors were investigated by Wang's group and found the receptor IFNGR1 co-expressed with neuronal cell adhesion molecule and gustducin expressing taste sensory cells. The presence of these receptors was corroborated by the phosphorylation of downstream STAT1 transcription activator. Administration of IFN has been shown to induce cellular death in taste buds (Wang, Zhou, Brand, & Huang, 2007).

1.11 CELLULAR DEATH

Understanding cellular death may help us understand more about how chemotherapy drugs such as cyclophosphamide affects the taste system. Cell death is an essential homeostatic mechanism in multicellular organisms which generally maintains the overall architecture by removing damaged cells that could be detrimental to the well-being of the organism. There exist three distinct modes of cell death: apoptosis, autophagic cell death and necrosis. Apoptosis is a controlled cell death process whereby the cells dismantle without causing any damage to the nearby tissues. Autophagy is more of a survival process where membrane engulfment along with catabolic degradation of cytoplasmic materials occur as a response of metabolic stress within cells. Necrosis is the form of cell death resulting from any cellular injury and toxin. It is characterized by loss of plasma membrane integrity and swelling. All the above-mentioned modes of cell death occur during different

phases of inflammation which could be the result of the cytokines inducing the immune cells to cause cell death. Understanding the nature of cell death following tissue damage can be quite important (Green & Llambi, 2015).

1.11.1 Apoptosis

Apoptosis, also known as programmed cell death, is characterized by cell shrinkage, blebbing, and condensation of chromatin ultimately leading to cell death via phagocytosis by macrophages. Apoptosis is a well-regulated process which, when it fails, causes serious pathological conditions. This is a caspase-mediated cell death. An initial understanding of apoptosis was investigated in *Caenorhabditis elegans* which precisely form 1090 cells of which 31 of them die post development. There are two modes of apoptosis which is mediated by different but converging pathways: a mitochondrial-mediated pathway (also known as the intrinsic pathway) and a death receptor pathway (Kerr, Wyllie, & Currie, 1972). The death receptor pathway is initiated by a ligand binding to membrane surface receptors such as CD95, TRAIL-R or TNFR1 that activates a downstream signaling pathway to recruit caspases. The membrane receptors such as TRAIL oligomerize after ligand binding to recruit Fas associated death domain and caspase-8 forming a complex called DISC. At this point effector caspases such as caspase 3, 7 and 9 are activated downstream of the complex. In contrast, the mitochondrial pathway is initiated by the loss of membrane integrity resulting in the release of cytochrome c which binds to Apaf-1 and procaspase-9 to form the apoptosome complex. The intrinsic or the mitochondrial pathway is initiated due to either DNA damage or cellular stress. Both pathways converge onto caspase-3 and other proteases to move the process of cell death ahead. The final step of the entire process involves the recognition of markers such as

phosphatidylserine to finally phagocytose the cell. Pro-survival signals regulate pro-apoptotic mechanism within cells. Bcl-2, IAP or NF-kB are just some of the protein molecules involved in the process (Green & Llambi, 2015; Yu et al., 2005; Zou, Henzel, Liu, Lutschg, & Wang, 1997)

1.11.2 Necrosis

Necrosis is an unregulated process and often can lead to an inflammatory condition in and around tissues. It is characterized by vacuolation of the cytoplasm and disintegration of plasma membrane. It is an energy independent process and the condition occurs when the metabolic processes within the cells break down. A very well-known example of when this process begins is during ischemia. Lack of oxygen, glucose and other factors could result in necrotic cell death in non-proliferating cells (Yuan & Kroemer, 2010)

Evidence suggests necrosis can be induced by microbial infection, reactive oxygen species (ROS), toxins, tissue injury and can be a component of normal physiological processes. Often when the small intestinal tissue is renewed both apoptosis and necrosis has been involved in cell death (Günther, Neumann, Neurath, & Becker, 2013). Studies to determine whether necrosis could be a possible method of cell death in tissue development, found that genetic deletion of caspase-3 and caspase-9 do not affect the neuronal loss in spinal cord. DNA alkylating agents can induce necrotic cell death initiated by PARP, in proliferating cells. The inflammatory component of necrotic cell death in tumors could induce an immune response which could result in an efficient tumor cell loss (Zong, Ditsworth, Bauer, Wang, & Thompson, 2004)

1.11.3 Autophagy

Autophagy is an evolutionarily conserved lysosome mediated pathway which has several important functions. It is important during remodeling and differentiation of tissues, produces amino acids during limited nutrient availability and gets rid of damaged organelles within cells. Autophagy is a stimulated process which means it is activated during oxidative stress, or the accumulation of misfolded proteins, hormonal signaling, and irradiation (Kroemer & Levine, 2008; Shen, Kepp, & Kroemer, 2012).

1.11.4 Summary on cell death

One of the common mechanisms by which chemotherapy drugs induce cellular damage is apoptosis. Cell death or apoptosis is induced via signaling pathways within the cells which are activated by Death Receptors, such as the TNF family receptors, resulting in caspase activation and recruitment. These processes finally converge at a single event, mitochondrial membrane permeabilization, which leads to cell death. Some of the cytotoxic agents directly affect the mitochondria by acting upon the bcl-2 family proteins or adenine translocase within these cells (Debatin, Poncet, & Kroemer, 2002).

1.12 RESEARCH QUESTION AND HYPOTHESIS

Chemotherapy-induced taste sensory loss is commonly documented in patients undergoing chemotherapy, resulting mainly in malnutrition. Some of the behavioral and cellular experiments in our lab suggested the loss of taste buds over a course of time has been responsible for an augmentation of taste detection threshold for common tastes. Cyclophosphamide is an alkylating agent which causes toxicity by disrupting the cell replication cycle. Studies in our lab suggest that cyclophosphamide has a toxic effect within

hours of administration and a second wave of toxic effects detectable within 18-24 hours, corroborated by TUNEL and caspase-3 assays (Mukherjee et al., 2017) These findings suggest that cyclophosphamide may cause an inflammatory response in the peripheral taste system. Previously, studies have suggested lipo-polysaccharide, an outer layer of the bacterial cell wall, mimics an infection that induces an inflammatory response in taste buds and expresses pro-inflammatory cytokines in taste buds (Cohn, Kim, Huang, Brand, & Wang, 2010; Wang et al., 2007) Our study explores the general hypothesis that a chemotherapy drug induces an inflammation which could be a result of the insult that chemotherapy-led toxicity imposes on the tissues.

More specifically, our investigation involves detection of Tumor Necrosis Factor-α (TNFα), a pro-inflammatory cytokine expressed in the taste buds present in both fungiform and circumvallate papillae.

My thesis is testing two hypotheses: 1) cyclophosphamide induces inflammation in taste sensory system, specifically detecting the expression of TNFα in either Type II or Type III cells within the taste buds, and 2) amifostine can protect the taste system from cyclophosphamide-induced inflammation by reducing the levels of TNFα at least 24-hour post CYP injection.

Our investigation involves Immunohistochemical labeling to detect TNF-α along with the respective taste cell marker within the taste bud to identify the taste cells over a period of 72 hours post-cyclophosphamide injection. A second component of this experiment involves pre-injection of amifostine and then cyclophosphamide followed by perfusion at 24-hour post injection and immunohistochemical labeling to determine the reduction in the overall TNFα levels in taste sensory cells.

CHAPTER 2: EXPRESSION OF PRO-INFLAMMATORY CYTOKINE DURING CHEMOTHERAPY IN TASTE BUDS AND AMIFOSTINE AS A CYTOPROTECTIVE AGENT

2.1 Introduction

The gustatory system is an important sensory system involved in distinguishing nutritious from poisonous substances. It also is involved in preparing the gut for the cephalic phase by secreting necessary enzymes and juices for the digestion of food. Any disturbance in the taste sensory system can be deleterious for the overall health and well-being of humans. Disturbance in taste could be a result of infection, drug-induced injuries, radiation, or other forms of insult (Chaudhari & Roper, 2010).

All these causes can have severe effects on gustation which can result in avoidance of food and potentially long-term malnutrition. Chemotherapy is a major cause of taste-related dysfunction in patients undergoing treatment (Comeau et al., 2001; Hong et al., 2009). Taste dysfunctions generally are side-effects of treating cancer in patients by targeting proliferating cells in different phases of replication cycles. However, these drugs are often known to be non-specific and do not differentiate between healthy and cancer-causing cells, thereby damaging healthy cells such as taste sensory cells (TSCs) along with the cancer-causing ones (Mukherjee et al., 2017).

Chemotherapy drugs can elevate taste detection thresholds for basic tastes. Of 518 patients in one study who received chemotherapy at different centers, 134 complained of taste sensory changes after a few weeks of treatment (Bernhardson et al., 2007; Bernhardson et al., 2008). Chemotherapy patients also exhibited taste sensitivity deficits

when tested with electrogustometric analysis. Electrogustometric analysis reliably tests the functional integrity of nerves, taste sensory cells, and the brain centers for proper functioning and taste detection (Berteretche et al., 2004). In addition, qualitative and quantitative assessment of taste in patients undergoing chemotherapy, were done using filter paper strips impregnated with basic taste substances. These patients also showed a transient loss of taste compared to healthy controls. This study also reported the effect of chemotherapy was more distinct on salt taste sensation than other basic tastes (Steinbach et al., 2009).

There appear to be various reasons for a loss in taste sensitivity. For example, cyclophosphamide (CYP), a well-established chemotherapy drug, raises taste thresholds because it can kill TSCs, damage or alter taste bud structure, and disrupt cell proliferation that is involved in replacing TSCs when they die (Mukherjee et al., 2017). Other secondary effects of chemotherapy or radiotherapy include reduced saliva output due to damage to the von-Ebner gland. Xerostomia can change taste thresholds due to lower production in saliva which is important in making the nutrient available to the taste bud apical ends (Mese et al., 2007; Mukherjee & Delay, 2011).

Research using behavioral assays with umami stimuli, found a biphasic pattern in taste dysfunction after a single dose of CYP. Detection of umami taste showed a decline in sensitivity during the first four days post injection and again beginning about eight days post injection. Cellular assays confirmed a decline in mean fungiform papillae, both with and without pores, 2-4 days post injection which did not recover for 12-16 days. In contrast, circumvallate taste buds began showing a decline by 7-8 day and began to recover 12 days

post injection (Mukherjee et al., 2013). A decline in the number of taste buds suggests a direct cellular effect of the chemotherapy drug.

TSCs are epithelial cells, present in onion shaped taste buds, and are responsible for taste detection and transduction downstream. TSCs are elongated cells spanning from the apical pore to the basal end of the taste bud structure. There are about 50-100 TSCs within the taste buds, and they can be of different types such as Type I, II, III and IV (Farbman, 1965; Finger, 2005). Type I cells are mature columnar shaped, generate protruding structures around other cell types, and act as a glial-like cell by expressing GLAST and EctoATPase for neurotransmitter clearance. Type II cells are large, having a round nucleus and expressing G protein-coupled receptors and downstream signaling machinery for sweet, bitter or umami taste transduction. Type III cells are slender in structure, characterized by synaptic contacts and the expression of synaptic membrane protein, SNARE complexes (DeFazio et al., 2006). These cells have a short life span ranging between 8 to 24 days with different cell types having differing life spans (Finger, 2005).

TUNEL and caspase-3 assays show CYP has a toxic effect on TSCs, causing cells to die at a very early stage post injection. TUNEL assays, which detect fragmented DNA, showed a peak response at 6-8 hours post-injection, whereas caspase-3 assays of apoptosis revealed a peak signal 18-24 hours post injection. Results from these assays suggest an early drug-related necrosis, followed by apoptosis in TSCs (Mukherjee et al., 2017). Similar data were reported for TUNEL and caspase-3 in bladder inflammation after CYP injection in rats (Jezernik, Romih, Mannherz, & Koprivec, 2003). CYP injections in rats have also resulted in a significant expression of cytokine mRNA in the bladder tissue

indicating an inflammatory response within the bladder (Jezernik et al., 2003; Malley & Vizzard, 2002).

Inflammation is an immune response to either infection, tissue damage or toxicity related to drugs and involves the expression of cytokines as an immune modulating factor (Vakkila & Lotze, 2004). Inflammation within sensory cells within taste buds have been shown to cause taste related disorders (Wang et al., 2009). Inflammation due to the expression of cytokines in taste buds could be involved in cellular death as demonstrated earlier in the activation of interferon signaling pathways. Administration of IFN-α and IFN-γ had significantly increased programmed cell death of taste cells (Wang et al., 2007).

Previous studies using lipopolysaccharide (LPS), a component of bacterial cell wall component which mimics infection model, have found expression of pro-inflammatory cytokines within the taste sensory cells. Six hours after injections of LPS, TNF-α (proinflammatory cytokine), IFN-γ and IL-6 (both anti-inflammatory) were found localized within subsets of taste sensory cells. Induced by lipopolysaccharide injection, TNF-α was found in the taste buds, co-localized and highly expressed in sweet and umami sensing TSCs (Feng et al., 2012). TNF-α is a trimeric protein molecule known to be released by immune cells such as monocytes, macrophages and activated by T lymphocytes. TNF-α induces its effect by binding the trimeric protein to two types of receptors, TNFR1 or TNFR2. TNF-α is known to have various purposes within the cells such as activating signaling pathways or inducing immune response and inflammation. It does have some protective effect, but with over-expression it could be deleterious to an extent that results in cell death. Also, its name comes from its ability to cause necrotic

cell death as documented in some tumor cells (Idriss et al.,2000). Prior work suggests it is released constitutively in cells even during normal physiological conditions, perhaps having broader functions in protecting cells (Feng et al., 2012).

In the present study, we looked at TNF-α in both fungiform and circumvallate papillae after CYP administration to determine if it was co-localized in specific taste sensory cells. TNF-α is a pro-inflammatory cytokine produced by immune cells such as macrophages, lymphocytes and monocytes inducing an inflammatory reaction during any abnormalities within the cells. It is also important in regulating multiple physiological processes such as differentiation, proliferation and cell survival. Any dysregulation in its normal cellular expression could affect multiple physiological processes and thus we decided to investigate its expression in its role as an inflammatory marker causing cellular damage. Therefore, our study is designed to determine whether CYP, an alkylating agent, induces an inflammatory response within the taste sensory system and the type of taste sensory cells most affected.

Given the potential disruptive effects of chemotherapy, it is important to try to protect the taste system from the effects of drugs such as CYP. Studies have shown the sulfhydryl drug, amifostine (AMF), has cytoprotective effects within taste papillae. AMF was reported to significantly reduce the effects of CYP, e.g., preventing the loss of papillae, when injected before CYP (Mukherjee et al., 2013; Delay et al., 2019). CYP also dramatically reduces the population of proliferating cells that replace cells within taste bud due to their short life spans and high turnover rate (Mukherjee et al., 2013; Cohn et al., 2010; Chaudhari & Roper, 2010). Pretreatment with AMF protected this critical population of proliferating cells (Mukherjee et al., 2013). Therefore, a second goal of this study was

to determine whether the pre-injection of AMF prevents CYP-induced inflammation within the taste sensory system.

2.2 Methodology & Materials

2.2.1 Ethical Consideration

The animal care and experimental protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Vermont under the protocol 14-003. All efforts were made to minimize suffering.

2.2.2 Animals

Male C57BL/6J mice were obtained from Jackson Laboratories (Stock No: 000664; https://www.jax.org/strain/000664; Bar Harbor, ME, USA) which were housed and acclimatized in the animal care facility for at least 7 days before perfusion and further experimental procedures were performed. The animals were typically between 25-30 grams and were housed in groups of 2-4. The mouse colony was maintained on 12/12 hr light and dark cycles. Food (Purina Chow, Prolab RMH 3000) and water was provided to the animals *ad libitum*.

2.2.3 Drugs and reagents

CYP (cyclophosphamide monohydrate, 97%) was obtained from Arcos Organics, New Jersey, USA. The 0.9% sodium chloride injection, USP was obtained from Hospira Inc (Lake Forest, Illinois). AMF (Amifostine-2-(3-Aminopropyl) aminoethyl phosphorothioate, 97%, Cat no. 1019406) was obtained from (USP reference standard, Rockville, MD). Lipopolysaccharides from E. coli 0111: B4 was obtained from Sigma Aldrich (L2630-10MG St. Louis, MO). All the drugs and reagents were freshly prepared in saline solution before injecting into the animals.

2.2.4 Dosage

The dose for CYP was chosen using a dose response curve where three different doses (37.5, 75 and 150) mg/Kg was used. It was observed that a dose of 37.5 mg/kg caused some difficult to detect damage to taste buds in mice. On the other hand, a dose of 150mg/kg causes nephrotoxicity in mice (Delay et al., 2019; Girard et al., 2011; Vizzard, 2000). Ultimately, a dose of 75mg/kg was chosen, since it has detectable taste-related changes within the taste buds.

The dose for AMF was based on clinical reports and previous work with mice. When used as a pretreatment to CYP, 100mg/kg AMF administered subcutaneously has a protective effect over the taste cells of mice without apparent side effects (Mukherjee et al., 2013). Previous clinical data suggest high doses can cause numerous side effects (Adamson et al., 1995). To be able to avoid these potential adverse effects and to be able to compare our results with previous data, a dose of 100 mg/kg was chosen for this study.

2.2.5 Tissue Collection

The animals for the experiment were randomly assigned to different time points prior to CYP or AMF injection. Post CYP injection, the animals were perfused at one of the following time points: 8 hr, 16 hr, 24 hr, 48 hr and 72 hr post injection. At the assigned time point they were euthanized using sodium pentobarbital. The control group was injected with saline (1 ml/kg) followed by immediate perfusion and was represented as 0 hr. A group of animals were injected with AMF, 30 minutes prior to CYP injection and perfused 24hr post injection. In addition, a control group was injected with AMF and perfused 24 hr post injection. After euthanizing the animals from each group with sodium pentobarbital, they were perfused with 0.1M phosphate-buffered saline (PBS) mixed with

heparin, followed by 4% paraformaldehyde mixed with PBS to immediately fixate the tongue tissues (Electron Microscopy Sciences, PA, USA). The tissues were then soaked in 4% paraformaldehyde solution for about 3 hours. Post paraformaldehyde fixation, the tongue tissue was transferred to 30% sucrose solution in PBS for at least two days before blocking them for storage in a cryo-mold using OCT. These were stored at -80^{o} C until cryosectioning. Tongue tissue was sectioned in a cryostat at 12µm thickness to obtain thin slices of tongue papillae which were mounted directly on Super-frost Plus glass slides (Fischer Scientific, MA, USA).

2.2.6 Immunohistochemical labeling

2.2.6.1 Co-labeling TNF-α with PLC. To Co-label TNF-α with PlCb2, tissues collected after injecting CYP, AMF and saline treatments were thawed and washed in PBS. Tissue processing was completed in a series of batches of slides. Each batch of tissue included sections representing at least one animal from each group injected with CYP and perfused at 0, 8, 16, 24, 48-, and 72-hour post injection. Each batch also included sections of an animal that was injected with AMF and CYP 30 minutes post AMF, and an animal that had received AMF alone. The slides were washed in 0.1M PBS before the antigen retrieval procedure in 10mM sodium citrate for 20 mins at 90 C. The slides were subsequently allowed to cool down for 40 mins before washing them in 0.1M PBS with 0.3% Triton-X. The slides were then incubated in a permeabilization buffer comprised of 0.1% Saponin (Cat no# SAE0073, Sigma Aldrich, St. Louis, MO, USA), PBS and 0.3% Triton-X 100 (Cat # T9284, Sigma Aldrich, St. Louis, MO, USA) for an hour. Subsequently, the slides were blocked using 5% Normal Donkey Serum, 0.1% Saponin and PBS buffer for 1 hour 30 minutes in room temperature. Primary antibodies against

TNF-α and PLCβ2 were applied using, goat anti-TNF-α antibody (R&D Systems, Cat no. AF510NA) and rabbit anti-PLCβ2 antibody (Cat # sc-206, Santa Cruz Biotechnology, Inc, Dallas, TX, USA) mixed in 5% normal donkey serum solution at 1:75 and 1:500 concentrations, respectively, for 24hrs at 4^{o} C. The slides were rinsed in PBS 0.3% Triton-X before applying Alexa Fluor 488 donkey anti-rabbit IgG (Cat #A21206) and Alexa Fluor 546 donkey anti-goat (A11056) secondary antibody (Life Technologies ltd., Rockville, MD, USA) for 1.5 hours at 1:1000 concentration. The slides were subsequently washed in 0.1 M PBS, incubated in DAPI, a nuclear stain to facilitate cell counting and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

2.2.6.2 Co-labeling TNF-α with SNAP-25. Slides were also labeled to detect TNF-α in Type III cells. Type III cells are identified with the biomarker SNAP-25 produced in rabbit (Cat # S9684, Sigma Aldrich, St. Louis, MO, USA). A similar protocol as the co-labeling of TNF-α with PLCβ2 was used to co-label SNAP-25 with TNF-α.

2.2.7 Image analysis and cell counts

Data collection was performed using fluorescence imaging with a Nikon Eclipse E600 fluorescent microscope with a photometric Cool SNAP EZ camera for image capturing. The images were captured using SPOT Advanced (Version 5.1) software keeping the same gain and a relatively similar exposure time. Subsequently, the images were sharpened, merged, and counted using Adobe Photoshop CS8 and NIH ImageJ software.

Taste buds on either side of the circumvallate papilla close to the trench were selected for analysis. A taste bud was selected only if the onion-shaped structure was maintained from the pore at the trench to the basal region of the bud and the cells were

oriented along their long axis. Fungiform taste buds were selected only if they had a taste pore and their cells appeared oriented along their long axis. Cells were labeled PLCβ2 or SNAP25, TNF-α, and DAPI in red, green and blue, respectively. For each animal, 7-10 taste buds were identified and counted for TSCs for each taste cell marker. Taste buds from four animals were selected for each time point and AMF condition.

Quantification of each taste bud included counts of total numbers of cells within a taste bud (DAPI), the total number of cells labeled with either PLCβ2 for Type II or SNAP-25 for Type III cells, the number of cells labeled for TNF-α, and the number of cells double labeled with TNF-α and either PLCβ2 or SNAP-25. To determine the percentage of each cell type also double-labeled with TNF-α, the total number PLCβ2 or SNAP-25 labeled cells was then used as the divisor of the cell count co-labeled with TNF-α, and then multiplied by 100. The proportions obtained are averaged across all four animals at different treatment conditions. The data obtained were analyzed on GraphPad Prism 8 where a one-way ANOVA was performed to compare treatment groups among each other to identify significance differences between groups. Tukey's multiple comparison tests were then used to identify which groups differed significantly. An alpha of $P < 0.01$ was used for all statistical comparisons throughout the experiment.

2.3 Results

Infection-induced inflammation is quite common in taste buds as described by the presence of pro-inflammatory cytokines (Wang, Zhou, Brand, & Huang, 2007). The purpose of our study was to detect whether inflammation is induced by CYP in the taste sensory organ and whether AMF can prevent this inflammation. We found that CYP

induces a higher expression of TNF-α in taste buds within fungiform and circumvallate papillae after a single CYP injection and AMF successfully prevents higher expression of TNF-α in taste buds, when pre-injected.

We had four different sets of data based on differences in the papillae type and the type of cellular marker labeling the two different types of TSCs. The four sets are Type II TSCs in fungiform taste buds, Type II TSCs in circumvallate taste buds, Type III TSCs in fungiform taste buds, and Type III TSCs in circumvallate taste buds. Each set included the data for the mice at each post injection time point. The data analyses are presented in the order of (1) total number of cells (DAPI signal), (2) cells labeled as TNF-α, (3) cells labeled as Type II or Type III, and (4) cells double labeled as a cell type+ TNF-α,

2.3.1. CYP induced expression of TNF-α

2.3.1.1. Type II Cells in Fungiform and Circumvallate Taste Buds.

Fungiform taste buds. The total number of cells (labeled with DAPI) observed in fungiform taste buds of the six CYP groups did not differ statistically (Figure 1A). The one-way ANOVA evaluation of the total number of cells labeled with TNF-α per taste bud showed significant differences in effects of CYP between groups ($F(5,18) = 33.20$, $P<0.0001$; Figure 1C, Figure 2). Tukey's multiple comparison shows a difference between 8hr versus saline, 16, 48, and 72hr groups ($P<0.005$). The total TNF-α per taste bud was higher at 8hr than all the other groups mentioned. A significantly higher number of TNF-α was also observed at 24hr than saline, 16, 48 and 72 hr groups of animals administered CYP ($P<0.002$). In addition, the one-way ANOVA applied to the number of PLCβ2 labeled cells (regardless of TNF-α labeling) identified no significant difference in effect of CYP over the time frame of the experiment ($F(5,18) = 0.866$, $P>0.01$; Figure 1B, Figure 2).

However, the percentage of Type II cells also exhibiting TNF-α label varied significantly between mice at each time point ($F(5,18) = 20.82$, $P<0.0001$; Figure 1D, Figure 2). Multiple comparison tests indicated that the percent of double-labeled cells were significantly higher at 8 and 24 hr than observed in saline, 16, 48, and 72 hr time points ($P<0.01$).

Circumvallate taste buds. The total number of cells (labeled with DAPI) observed in circumvallate taste buds of the six CYP groups did not differ statistically and were quite constant across groups (Figure 3A). The one-way ANOVA evaluation of the total number of cells labeled with TNF-α showed significant differences between CYP groups ($F(5,18) = 25.39$, $P<0.0001$; Figure 3C, Figure 4). Tukey's multiple comparison tests showed a difference in the TNF-α cells between 8hr and 24hr versus saline, 16, 48, 72hr CYP groups ($P<0.0006$). The TNF-α cells per taste bud was much higher in animals at 8hr and 24hr than at 16, 48 and 72 hr CYP groups. In addition, the one-way ANOVA applied to the number of PLCβ2 labeled cells (regardless of TNF-α labeling) identified no significant effect of CYP across groups over the time course of the experiment ($F(5,18) = 0.86$, $P=0.5$; Figure 3B, Figure 4). However, the percent of Type II cells also exhibiting TNF-α label varied significantly between groups at each time point ($F(5,18) = 11.92$, $P<0.0001$; Figure 3D, Figure 4). Multiple comparison tests indicated that the percent of double-labeled cells were significantly higher at 8 and 24 hr than observed in saline, 16, 48, and 72 hr time points ($P<0.01$).

2.3.1.2. Type III cells in Fungiform and Circumvallate taste buds

Fungiform taste buds. Our observations did not show any statistical difference in the total number of cells (labeled with DAPI) observed in fungiform taste buds of the six

CYP groups, with cells evenly distributed among each of the six groups (Figure 5A). The one-way ANOVA evaluation of the average number of cells labeled with TNF-α also showed no significant difference in the effect of CYP over groups ($F(5,18) = 2.136$, $P<0.1$); Figure 5C, Figure 6). In addition, the one-way ANOVA applied to the number of SNAP-25 labeled cells (regardless of TNF-α labeling) identified no significant difference in effect of CYP over the time frame of the experiment ($F(5, 18) = 2.403$, $P<0.1$; Figure 5B, Figure 6). The average number of Type III (SNAP-25) cells remained quite constant throughout all the groups. Similarly, the number of Type III cells exhibiting TNF-α label did not vary significantly between mice at each time point ($F(5,18) =0.7449$, $P<1$; Figure 5D, Figure 6).

Circumvallate taste buds. The total number of cells (labeled with DAPI) observed in circumvallate taste buds of the six CYP groups did not differ statistically and the average numbers were quite even (Figure 7A). The one-way ANOVA evaluation of the total number of cells labeled with TNF-α show significant difference in effect of CYP between groups, ($F(5,18) = 7.398$, $P<0.001$; Figure 7C, Figure 8). Tukey's multiple comparison tests identified differences between the 24 hr group and 48 hr (0.001). The total number of TNF-α per taste bud was considerably higher at 24 hr than 48 hr group. Similarly, a statistical difference was observed between 8hr and the 48hr CYP group, where the total TNF-α per taste bud at 8hr was higher than the numbers observed at 48hr ($P<0.01$). In addition, the one-way ANOVA applied to the number of SNAP-25 labeled cells (regardless of TNF-α labeling) identified no significant effect of CYP over the time frame of the experiment, with an equivalent number of cells across all groups ($F(5, 36) =0.1209$, $P=0.9$; Figure 7B, Figure 8). However, the percent of Type III cells also labeled with TNF-α

antibody varied significantly between mice at each time point ($F(5,18) = 5.699$, $P<0.0001$; Figure 7D, Figure 8). Tukey's multiple comparison tests indicated that the percent of SNAP-25/TNF-α positive cells were significantly higher at 24hr than saline and 48hr groups. A significant difference was also observed between 8hr and 48hr groups, where the number of Type III cells with TNF-α label at 8hr was higher than at 48hr ($P<0.01$).

2.3.2. Amifostine

2.3.2.1. Type II (Fungiform, Circumvallate). Fungiform taste buds: The average number of DAPI labeled cells in the fungiform taste buds did not differ statistically among the four different groups either injected with saline, CYP-24hr and/or AMF-24hr (Figure 9A). In addition, the one-way ANOVA evaluation of the TNF-α labeled cells ($F(3,12) = 25.68$, $P<0.0001$; Figure 9C, Figure 10) detected significant differences among treatment groups. The mean TNF-α labeled cells per taste bud was significantly higher in the CYP treated group than saline, AMF and CYP-AMF treated groups ($P<0.01$). One-way ANOVA evaluation of PLCβ2 cells (regardless of TNF-α labeling) ($F(3,12) = 0.5878$, $P>0.1$; Figure 9B, Figure 10) in the taste buds did not detect any statistical difference among its groups. The number of PLCβ2 cells were quite constant across groups. However, the percent of Type II cells double-labeled with TNF-α antibody varied significantly between treatment groups ($F(3,12) = 13.82$, $P<0.01$; Figure 9D, Figure 10). Tukey's multiple comparisons of the treatment groups suggest a significantly higher percentage of PLCβ2 and TNF-α double-labeled cells in the CYP group than in the saline, AMF, and AMF-CYP treatment groups (all $Ps<0.01$).

Circumvallate taste buds: The average number of DAPI labeled cells in the circumvallate taste buds did not differ significantly among different treatment groups

(Figure 11A). The one-way ANOVA evaluation of the average TNF-α labeled cells in the circumvallate taste buds exhibit a significant difference among its treatment groups ($F(3,12) = 19.52$, $P<0.01$; Figure 11C, Figure 12). Tukey's multiple comparison tests found a significantly higher average number of TNF-α cells in the CYP group compared to saline, AMF, and AMF-CYP groups. Similarly, a one-way ANOVA evaluation of the average number of PLCβ2 positive cells in the taste buds (regardless of TNF-α labeling) found significant differences between its treatment groups ($F(3,12) = 4.844$, $P<0.01$; Figure 11B, Figure 12). Tukey's multiple comparison tests comparing these groups revealed significantly and unusually higher number of PLCβ2 cells in CYP than in the AMF-CYP groups. At the same time, the percent of Type II cells co-labeled with TNF-α showed a significant difference among its groups, ($F(3,12) = 16.57$, $P<0.01$; Figure 11D, Figure 12). Tukey's multiple comparisons tests detected a significantly higher percentage of double-labeled cells in the CYP group of animals than the rest of the groups ($P<0.001$).

2.3.2.2. Type III (Fungiform, Circumvallate). Fungiform taste buds: The average number of DAPI labeled cells in the fungiform taste buds did not differ statistically among the four different groups either injected with saline, CYP and/or AMF (Figure 13A). The one-way ANOVA evaluation of the sum of TNF-α labeled cells in each taste buds showed no significant statistical difference among its groups ($F(3,12) = 1.555$, $P>0.1$; Figure 13C, Figure 14). At the same time, the one-way ANOVA evaluation of SNAP-25 cells in the taste buds (regardless of TNF-α labeling) indicated no statistical difference ($F(3,12) = 1.551$, $P=0.252$; Figure 13B, Figure 14). Also, the percentage of Type III cells exhibiting TNF-α label did not vary significantly between treatment groups ($F(3,12) = 0.6582$, $P=0.593$; Figure 13D, Figure 14).

Circumvallate taste buds: The average number of DAPI labeled cells in the circumvallate taste buds did not differ significantly among different treatment groups (Figure 15A). However, the ANOVA evaluation of the average TNF-α labeled cells in the circumvallate taste buds showed an overall significant difference among its treatment groups ($F(3,12) = 6.838$, $P<0.01$; Figure 15C, Figure 16). We detected a significantly higher average TNF-α labeled cells in CYP injected group than the AMF group. Similarly, the one-way ANOVA evaluation of the average SNAP-25 cells in the taste buds (regardless of TNF-α labeling) show no significant difference between its treatment groups ($F(3,12) = 2.193$, $P>0.01$; Figure 15B, Figure 16). In contrast, the percent of Type III cells labeled with TNF-α showed a significant difference among treatment groups ($F(3,12) = 13.17$, $P<0.001$; Figure 15D, Figure 16). Tukey's multiple comparisons evaluation showed significant higher percentage of double-labeled cells in the CYP group compared to the other groups ($P<0.01$).

2.4 Discussion

Prior research has pointed both towards a behavioral and a cellular aspect of taste related dysfunction following administration of CYP. Early research indicated that CYP induced conditioned taste aversion, a very well-known phenomena of learning where individuals start avoiding food due to a prolonged nausea during administration of the drug which is associated with the ingestion of food. Even though the nausea is potentially an effect of the drug, it quickly gets associated with food. More recently, our lab has identified the loss of murine taste buds in both fungiform and circumvallate over a period of 16 days after CYP treatment. We have observed a loss in fungiform taste buds at around 2-4 days post CYP injection and a later phase in the loss of circumvallate papillae between 8-10

days post CYP-injection. Our present study has been an extension of the broader concept to find out if inflammation induces an immediate effect of the administration of CYP. This corroborates the behavioral study in our lab, which demonstrated bi-phasic increase in taste threshold at similar times (Mukherjee & Delay, 2011).

Our research supports our hypothesis that CYP induces an inflammatory response. Our findings suggest CYP induced differential expression of cytokine TNF-α in the taste buds of both fungiform and circumvallate papillae. CYP is known to have toxic effects within the taste sensory system and therefore we expected it to induce an inflammatory response immediately after its injection into the animals.

The expression of cytokines within the taste sensory system is constitutive and is a normal physiological phenomenon, providing either a protection or having a deleterious effect (Feng et al., 2012; Hsu, Xiong, & Goeddel, 1995). Cytokines have both paracrine and autocrine effect over the sensory cells, regulating important taste sensory mechanisms. We chose TNF-α as a cytokine marker due to its role in chronic inflammation in tissues, including the taste sensory system, which is induced by NF-kappaB and its ability to modulate numerous other molecules including cyclooxygenase (cox-2), inducible nitric oxide (iNOS), other inflammatory cytokines, and chemokines (Aggarwal, 2004; Ahn & Aggarwal, 2005; Cohn et al., 2010). It is also known to induce cell death within the tissues, by activating certain downstream cell death signaling pathways.

There are three different types of taste sensory cells, Types I, II, and III, responsible for taste detection. Our study involved investigating the expression of TNF-α in Type II and Type III cells after CYP administration but not the Type I cells. Type I cells are salt taste sensing cells and are difficult to quantify due to its unusual morphology and its

wrapping around other two types of taste cells, performing its glial like function. Type II cells are known to express TNF-α constitutively as well as when induced by lipopolysaccharide administration, whereas no evidence of expression was observed in Type III cells (Feng et al., 2012). Our study detected the expression of TNF-α in both Type II and Type III cells in taste buds, even though differentially based on papillae type. Type II cells were detected in both fungiform and circumvallate taste bud express TNF-α, whereas Type III cells expressed TNF-α only in the circumvallate taste buds. The presence of TNF-α in Type III cells suggest that there are signaling pathways present in these cells to induce an expression of TNF-α.

Earlier work in our lab using TUNEL and caspase-3 assays to investigate cell death after CYP administration. The TUNEL assay found cells dying at 4 hours, peaking at 6-8 hours, and returning to control levels after 18 hours. On the other hand, caspase-3 signal began to increase at 8 hours, peaked at 24 hours, and returned to normal levels by 48 hours post injection. Mukherjee et al. (2017) suggested CYP initially induces cell necrosis followed by apoptosis within hours after administration. Our study also supports these earlier findings. The peak expression of the cytokine, TNF-α in Type II cells was observed at 8- and 24-hour post CYP-injection which coincides with peak times of cell death in taste buds (Mukherjee et al., 2017). In contrast, increased expression of TNF-α in Type III cells was only observed 24 hours post injection indicating a late onset of any effect CYP could have over these cells. It may indicate the expression of pro-inflammatory cytokine TNF-α could have a role in cell death.

Our findings suggest that even a single administration of CYP can have a profound effect on the taste system within hours of administration. For example, the data for each of

our animals are based on a sample from a 12-um thick section typically with 15-20 DAPI labeled cells. If one assumes an individual taste bud is comprised of about 100 TSCs, then about 30 cells are of Type II and 10 cells are Type III. The data of our saline injected mice indicated about 20 percent of Type II TSCs in both fungiform and circumvallate taste buds also expressed TNF-α. Extended to our hypothetical bud of 100 cells, this means roughly 6 TSCs out of 30 in the taste bud. In contrast, CYP injected animals exhibited about 80 percent of Type II cells with TNF-α expression at 8- and 24-hours post injection. This suggests about 24 of 30 TSCs in the hypothetical taste bud showed increased TNF-α expression at these post injection periods. Similarly, saline injected mice in Type III cells only have about 20 percent of their TSC population express TNF-α, which stands roughly at around 2 cells per taste bud. On the contrary, the CYP injected animals have an expression of TNF-α in 6 TSCs out of 10 Type III cells in the entire taste bud. Extending these estimates, it is easy to see CYP-induced inflammation could inevitably have a significant impact over the entire taste system. Subsequent pro-inflammatory responses in the TSCs could lead to severe long-term implications in the taste sensory system of chemotherapy patients possibly resulting in taste sensory loss.

It is unlikely that saline could induce over-expression of TNF-α in the TSCs evaluated in this experiment. Saline was used as the vehicle of drug administration for both CYP and AMF. If saline is responsible for the TNF-α over-expression, then the effect would be visible in both CYP as well as AMF injected animals. However, the reduction of TNF-α in the TSCs pre-injected with AMF argue that TNF overexpression by saline would be a highly unlikely event.

We also analyzed the average number of cells present in taste buds which was DAPI labeled, corresponding all the cells in taste buds and the average Type II, Type III cells in the taste buds. As per our earlier study on cell death assays, we expected to see some decline in the overall number of cells in taste bud. We did see some decline in Type II cells in both fungiform and circumvallate taste buds at around 72-hour post injection, though it wasn't significantly different from the control groups. Similar observation was made for Type III cells in circumvallate papillae with no statistical difference. It is possible that the post-mitotic immature Type IV cells have already migrated into the taste bud which rapidly mature and differentiate into different types of taste sensory cells to replace the dying cells within the taste bud. The lack of decline in the overall DAPI labeled cells could also be the result of the counter expression of anti-inflammatory cytokines such as IL-10 in taste sensory cells. This could regulate the deleterious effect of pro-inflammatory cytokine TNF-α, which has the potential to induce cell death (Feng et al., 2014).

The second goal of our study was to determine if AMF could prevent an inflammatory response in taste sensory cells against the effect of CYP. AMF is a clinically proven drug, effective in protecting normal cells against radiation or chemotherapeutic effect. Clinical studies using AMF in patients undergoing cisplatin or CYP treatment to protect against chemotherapeutic induced damage of normal tissue, have been well documented in patients with ovarian carcinoma and have been effective in recovering a steady platelet count and preventing peripheral neuropathy (Rose, 1996). Importantly, AMF does not interfere in the efficacy of chemotherapy, vital to its use in patients with cancer (Capizzi, 1996).

Use of AMF in our study is warranted in view of its earlier use in patients to treat CYP induced hemorrhagic cystitis, a form of inflammation, an occurrence after bone marrow transplantation (Haldar, Dru, & Bhowmick, 2014; Srivastava et al., 1999). Extensive laboratory research on this drug has proved its efficacy in protecting taste sensory cells as well (Mukherjee et al., 2013). The mechanism of action of AMF suggests that it preferentially protects normal cells but not tumor cells due to its ability to differentiate the pH of these cells. This drug only metabolizes into its active compound in cells in which alkaline phosphatase is available in sufficient quantities (Grdina et al., 2000).

To our knowledge, no previous study has been conducted to investigate if AMF can block inflammatory cytokine TNF-α expression in the taste sensory system. We administered AMF at 100mg/Kg, pre-injecting it 30 minutes prior to CYP injection. The dosage administered was significantly less than what is usually utilized in patients with ovarian carcinoma (Rose, 1996). Our results indicate AMF has a protective effect in preventing CYP-induced inflammation, as evident by its ability to prevent increased expression of TNF-α in different taste sensory cell types within both the circumvallate and fungiform papillae. It was more effective in preventing an inflammatory response within the circumvallate than the fungiform papillae. The Type II cells in both the fungiform and circumvallate papillae showed a decline in expression of TNF-α in animal groups pre-injected with AMF than when only injected with CYP (Figures 10 & 12) On the other hand, Type III cells showed a greater decline in the inflammatory response within the circumvallate taste buds than in fungiform taste buds.

AMF seems to be a promising drug in preventing the inflammatory response within the taste cells. AMF's mode of action is to act as a scavenger to get rid of free radicals

produced during cellular stress and damage. It is quite possible these free radicals in cells are involved in triggering a cytokine response and inflammation. The metabolized active compound of AMF, possibly gets rid of the free radicals in cells to prevent the signaling mechanism to initiate and prevent an inflammatory response in cells.

Our study involves a holistic approach towards research on chemotherapy induced inflammation and ways to prevent it by using another drug. The cytokine we chose to investigate is well documented within the taste buds which performs functions such as cell proliferation and growth. Any unwanted upregulation can have deleterious consequences in the taste sensory system. This research is only the beginning of exciting new possible work that could be performed in the future to understand more about the consequences cytokines could have over the taste sensory system. Possible investigation into the other commonly found cytokines in the taste buds could reveal more about its functions in regulating the taste sensory system. Future studies could be conducted to corroborate our work by conducting conditioned knockout of the TNF-α receptor gene to visualize if the effect was due to the expression of TNF-α or other factors could be involved in it. Understanding inflammation is crucial for the cancer patients who regularly undergo chemotherapy to experience chemotherapy induced taste dysfunction so that anti-inflammatory cytokine therapy could be developed to reduce or ameliorate the effect of CYP induced taste related dysfunction.

CHAPTER 3: CONCLUSION AND FUTURE DIRECTIONS

3.1 CONCLUSION

Our research expands on a long-standing work started several years earlier in our lab on the effects of the chemotherapy drug, cyclophosphamide (CYP) on the taste system. Our earlier studies have shown an immediate effect of CYP (75mg/Kg) injection in animals which results in cell death peaking 8 hours post injection followed by another phase of cell death peaking at 24 hours post injection. This had initiated a cascade effect resulting in a biphasic deficit in taste function at 2-4 days and 8-12 days post CYP injection, the timing of which is associated with a loss in taste buds both in fungiform and circumvallate papillae.

Our present study tries to unravel some of the initial effects such as inflammation occurring immediately after CYP administration, that could be responsible for the toxicity induced cellular damage. For this work, we decided to investigate the presence of TNF-α in Type II and Type III taste sensory cells. TNF-α is a pro-inflammatory cytokine produced by immune cells such as macrophages, lymphocytes and monocytes inducing an inflammatory reaction to any abnormalities within the cells. It is also important in regulating multiple physiological processes such as differentiation, proliferation and cell survival. Any dysregulation in its normal cellular expression could affect multiple physiological processes and therefore we decided to investigate TNF-α expression in its role as an inflammatory marker after CYP administration.

Our study found expression of the cytokine TNF-α in the Type II and Type III taste sensory cells in taste buds of both circumvallate and fungiform papillae. Type II cells were most affected as we found a higher percentage of double-labeled cells in both circumvallate and fungiform papillae after CYP injection. Similar to previous research in which LPS was used to induce inflammation in the taste system of mice, there was increased expression of TNF-α in Type II cells after CYP injections, suggesting that one of the effects of CYP results in activating the signaling pathways for TNF-α in these cells. Our study also identified the presence of TNF-α in Type III cells which previously was not identified. Perhaps, CYP induces or activates another pathway which has not been identified yet.

Our second goal for this study dealt with the use of AMF, a sulfhydryl drug previously found to have cytoprotective effects against chemotherapy and radiotherapy in both laboratory and clinical settings. It is an FDA approved, widely used cytoprotective agent. Our investigation was to determine if pre-injection of AMF could prevent an inflammatory response within the Type II and Type III taste sensory cells. Indeed, we found a decline in cytokine TNF-α expression in both types of taste sensory cells after CYP administration. The decline in the expression of TNF-α was much more distinctive in the circumvallate than in the fungiform papillae. The cytokine response is due to the free radicals generated due to the toxicity caused by CYP. The free radicals are known to cause damage to the DNA by cross linkages in the single stranded DNA and amifostine likely helps to reduce the free radicals formed.

3.2 FUTURE DIRECTION

3.2.1 Does CYP induced inflammation in taste sensory cells affect the Ki67 labeled proliferating cells

The effect that CYP has over cell proliferation over time within the taste buds is quite well studied in our lab. Observations made over a period of 16 days after CYP injection has shown a sharp decline in Ki67 positive cells (a cell cycle marker) within the basal layer of the taste bud up to 6 days post injection in both fungiform and circumvallate papillae (Delay et al., 2019; Mukherjee et al., 2013). This clearly suggests the taste sensory cell proliferation and replacement cycle is affected post CYP injection. Subsequent study to see the effects of CYP administered by dose fractionation has also indicated a suppression in Ki67 positive cells in the taste buds which does not completely recover until day 10 post injection (Delay et al., 2019).

In future, we could design a long-term study over a period of 16 days for both a single CYP injection and dose fractionation experiment to investigate the expression of pro-inflammatory cytokines as well as anti-inflammatory cytokines. This could bring to light, exactly if long term expression of pro-inflammatory cytokine could possibly suppress the cell proliferation cycle over an extended period. The purpose of looking into the anti-inflammatory cytokines would provide some clues into if their expression is a mode utilized by the taste sensory system to protect itself against toxicity of the drug as well as the release of pro-inflammatory cytokines.

3.2.2 Characterizing the specific subset of Type II cells affected by CYP induced over-expression of TNF-α?

In our current study we were able to determine that the Type II cells expressed the cytokine TNF-α in both fungiform and circumvallate taste buds. This peak expression was detected at 8 and 24-hour post-CYP injection. We conducted our study using only PLCβ2 to label all of the Type II cells. This leaves the study incomplete in terms of specifically identifying the subsets of Type II cells which could be most affected. In her earlier research, Wang had noticed only a subset of Type II cells expressing TNF-α, specifically Type II cells with T1R3 receptors. T1R3 receptors form heterodimer with T1R1 or T1R2 to detect umami or sweet substances. They have speculated the expression of TNF-α is primarily to regulate signaling pathways involved in these basic taste detection (Feng et al., 2012). We expect the primary function of TNF-α is to regulate taste pathways and may be found in only a subset of Type II cells.

For example, one immunohistochemistry experiment could be setup by using antibody labels against TNF-α, PLCβ2 and gustducin to determine if both subsets of cells are affected by CYP-induced inflammation. Gustducin is known for labeling a specific subset of Type II cells that detect bitter (Yang, Tabata, Crowley, Margolskee, & Kinnamon, 2000). The experiment could be conducted over a period of 72 hours along with saline control animals. Any co-expression of TNF-α with gustducin and PLCβ2 would suggest the subset of Type II cells are affected indicating its relevance and importance in regulating in all subsets of Type II cells. Conversely, a lack of co-expression would suggest a different subset of Type II cells are expressing TNF-α.

3.2.3 Does chemotherapy induced inflammation have any effect over the nerve endings adjacent to the taste buds?

Taste sensory disorders due to inflammatory infection has been well documented in previous work and patient accounts (Mann, 2002). A recent study suggested that LPS induced infection activates the interferon pathway in mice (Wang et al., 2007). Loss of taste buds along with rapid expression of cytokines IL-6 or TNF-α have also been observed in mice with inflammation (Cohn et al., 2010). All the above studies have suggested an adverse effect of inflammation on the functioning of the taste sensory system. Our lab has previously conducted behavioral studies in mice to observe taste acuity in umami taste after CYP-injection, showing a biphasic response between 2-4- and 8-12-days post injection. Significant losses in sensitivity to umami, salt and sucrose substances were observed (Mukherjee et al., 2013, 2017).

From our research work, we were able to identify the type of taste cells, most affected or inflamed due to chemotherapy. We found Type II cells in fungiform and circumvallate papillae to be inflamed as well as Type III cells in the taste buds of circumvallate papillae. The possible reasons for the elevation in taste detection thresholds for the basic tastes we have studied could be due the inflammation in different types of taste cells at an early stage due to chemotherapy resulting in an early cell death and loss of taste buds at a later stage.

It would be worthwhile to investigate any effect inflammation has over the nerve endings adjacent to the taste buds. It is also possible that encoding processes may be adversely affected by CYP-inflammation. Immunohistochemistry experiments could be setup using PGP 9.5 to label the nerve endings in control versus CYP injected animals to

see if CYP causes damage to the nerve endings that corresponds to the immediate effect of inflammation.

3.2.4 What are the other different types of cytokines expressed in taste buds due to chemotherapy induced inflammation?

This is an important study to identify other different types of cytokines that might be released by the taste buds due to chemotherapy administration. As I mentioned repeatedly in my earlier sections, the Wang laboratory has determined the presence of multiple pro and anti-inflammatory cytokines in the taste buds activated by the administration of LPS. The expression of both pro and anti-inflammatory cytokines indicates regulation of physiological consequences within taste buds. The anti-inflammatory cytokines could be a result of the over-expression of pro-inflammatory cytokines in the taste sensory system.

Similar in-situ or immunohistochemistry experiments could be setup to identify the presence of other cytokines such as IFN, IL-6 or IL-10 co-expressed in different types of taste cells. Most of our studies have investigated Type II and Type III cells in taste buds because quantifying Type I cells are difficult. But it would be worthwhile if we could devise methods to identify the expression of cytokines in Type I cells as well as it has important glial like properties and would be crucial to see if its overall function in maintaining its function in taste buds are affected or not.

3.2.5 Does dose fractionation have differential effect in the expression of cytokines in taste buds?

Future studies could include working with dose fractionation of chemotherapy drugs and observing the inflammatory effect it could have over the taste buds. Past research on single CYP administration versus dose fractionation of CYP on mice have resulted in

prolonged suppression of cell proliferation in mice after a dose fractionation regime. Fractionated doses also had a greater effect on mature cells by reducing its overall number in taste buds (Delay et al., 2019).

A similar dose fractionation immunohistochemistry study could be performed to investigate the expression of cytokines in taste buds and quantify the overall number of cells present in the taste buds in each group at different time points throughout the experiment compared to a single injection in mice. At the same time, qPCR could also be performed within each group of animals to quantify the level of expression in different taste sensory cell type within each group in dose fractionated CYP animals versus single CYP injection animals.

3.2.6 What is the basis of the TNF-α expression in Type III cells?

Prior research on the localization of cytokines, especially TNF-α, was unable to detect them in Type III cells. They were only localized to a specific subset of Type II cells (Feng et al., 2012). Our research determined the presence of TNF-α in Type III SNAP-25 labeled cells. It could indicate the presence of another signaling pathway distinct from Type II cells activating downstream inflammatory pathway, involved in some regulatory mechanism in Type III taste sensory cells.

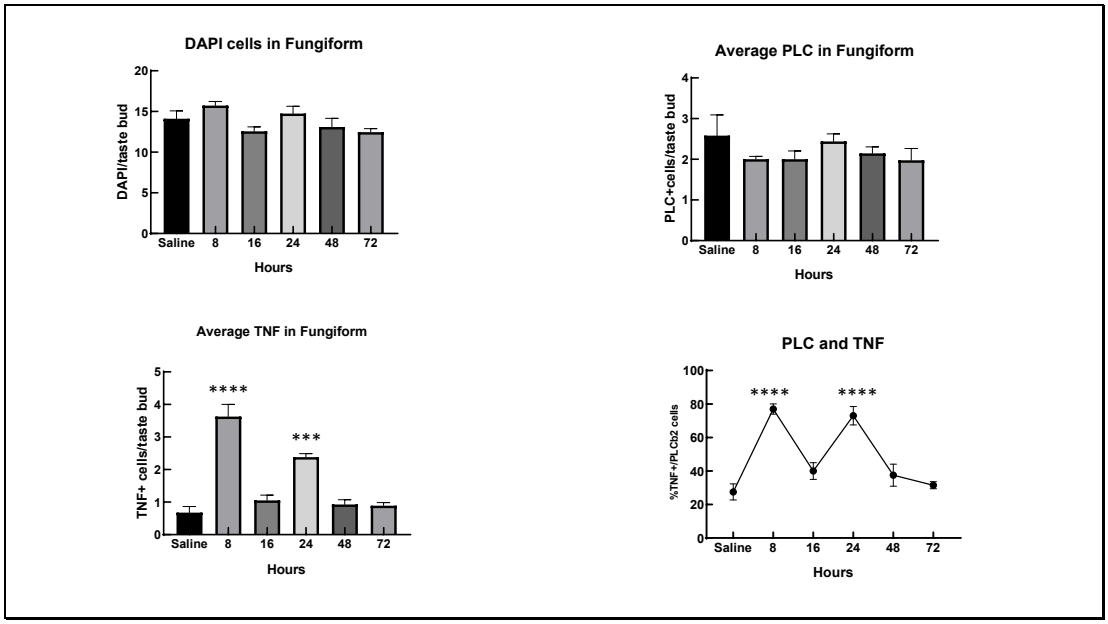
It would be worthwhile to identify the cell signaling pathway involved in Type III taste sensory cells within the taste buds using immunohistochemistry and immunofluorescent proteins. These methods may help to localize some possible receptors involved in detecting CYP as a ligand activating downstream signaling pathways and visualizing it under the microscope. Another method that could be used would be western blot. For example, to detect tyrosine phosphorylated protein that could be possibly

involved in the signaling pathway, we could run lysate and detect it primary and secondary antibody against those proteins.

3.3 Summary

Our study is quite important in addressing taste related disorders patients encounter when undergoing chemotherapy on a regular basis. It is vital to the survival and recovery of these patients from cancer, lessening the persistent suffering they face. Our study simply deals with one of the many factors associated with chemotherapy treatment. We have had promising results in identifying potential deleterious effects of CYP on taste buds that has resulted in broadening our curiosity and posing new questions which could be investigated. At the same time our Amifostine findings bring new light about the efficacy of this drug in reducing inflammation in the taste sensory system.

FIGURES



P** <0.0001**

P*<0.001**

Figure 1: Type II cells in fungiform taste buds. Top left to right: A) The bar graph illustrates the total number of cells per taste bud labeled in DAPI remained constant across 72 hours. B) Mean number of PLCβ2 labeled cells per taste bud in mice over a period of 72 hours. Bottom left to right: C) Bar graph indicates the mean number of TNF-α positive cells per taste bud in mice after CYP was administered showed peak responses at 8 and 24 hours post CYP-injection in mice. D) The line graph illustrates the mean % TNF-α positive cells co-labeled with PLCβ2, peaked at 8 and 24 hours in mice over a period of 72 hours, administered CYP (n/group=4).

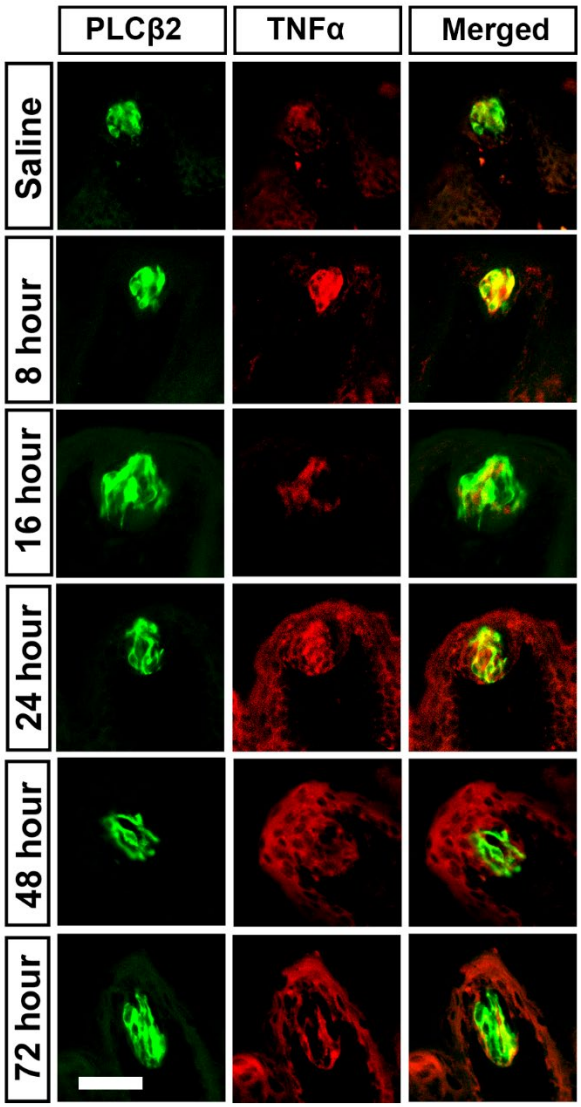
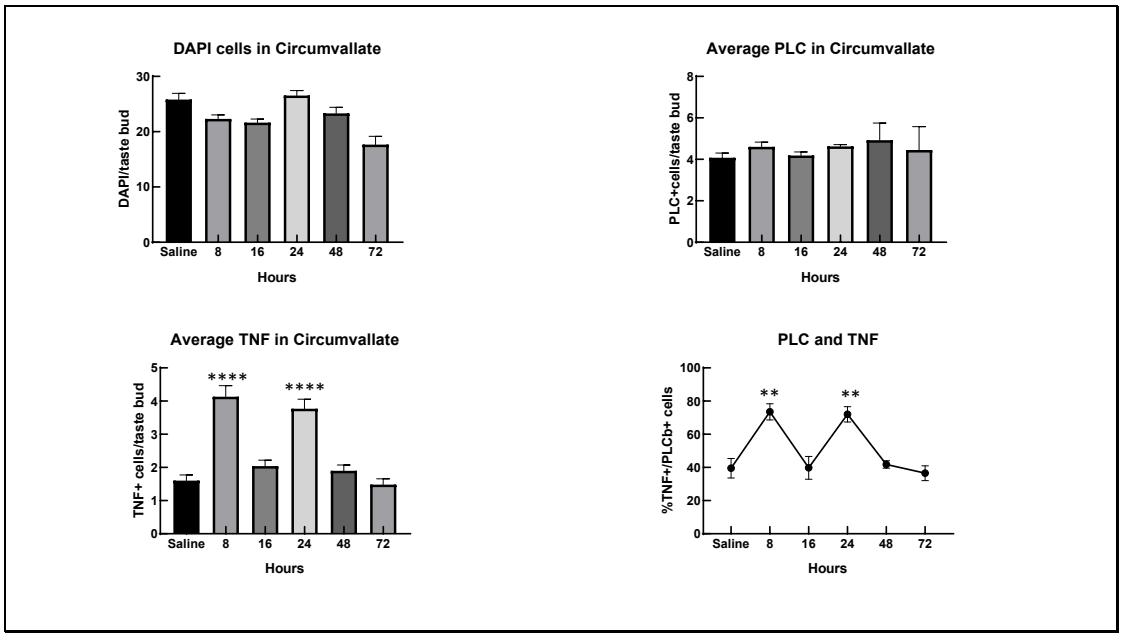


Figure 2. Visualization of Type II cells in fungiform taste buds from mice at 0, 8, 16, 24, 48, and 72 hours post CYP injection. Columns A, B, C show isolated taste buds labeled with PLCβ2 (Green), TNFα (Red), and cells co-labeled with TNFα and PLCβ2, respectively. TNFα expression peaks at 8 hours and 24 hours post-injection. The graphical data show a peak inflammatory response at 8 hr and 24 hr, respectively (n/group=4; scale bar = 20μm).



P<0.01**
P**<0.0001**

Figure 3: Type II cells in circumvallate taste buds. From top left to right: A) The bar graph illustrates the total number of cells per taste bud labeled with DAPI remained constant across 72 hours. B) The mean number of PLCβ2 labeled cells per taste bud in mice over a period of 72 hours. Bottom left to right: C) Bar graph indicates the mean number of TNF-α positive cells per taste bud in mice after CYP was administered showed peak response at 8 and 24 hours post CYP-injection in mice. D) The line graph illustrates the mean % TNF-α positive cells co-labeled with PLCβ2, peaked at 8 and 24 hours in mice over a period of 72 hours after administered CYP (n/group=4).

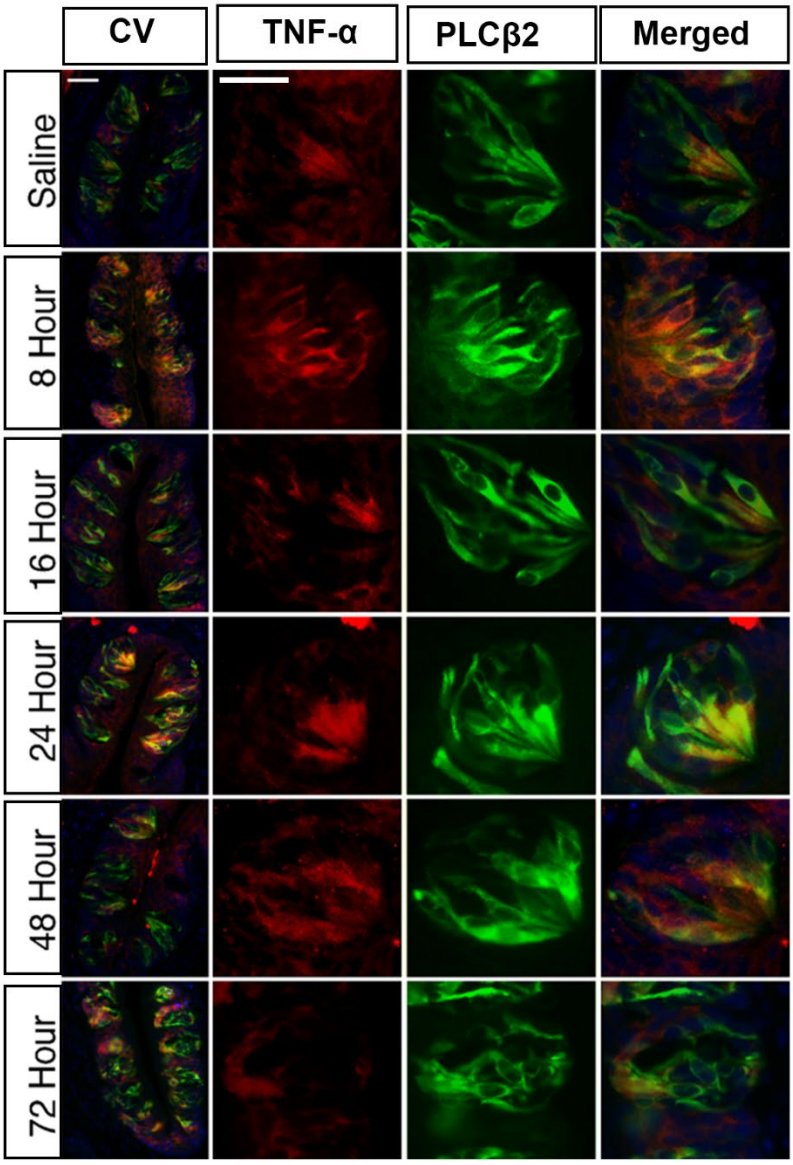


Figure 4. Visualization of Type II cells in circumvallate taste buds from mice at 0, 8, 16, 24, 48, and 72 hours post CYP injection. Column A shows a composite image of taste buds in a circumvallate trench (CV), showing both TNF-α and PLCβ2 merged. Columns B, C, D show isolated taste buds labeled with TNF-α (Red), PLCβ2 (Green), and cells co-labeled with TNF-α and PLCβ2, respectively. TNF-α expression peaks at 8 hours and 24 hours post- injection. The graphical data show a peak inflammatory response at 8 hr and 24 hr, respectively (n/group=4; scale bars = 20μm).

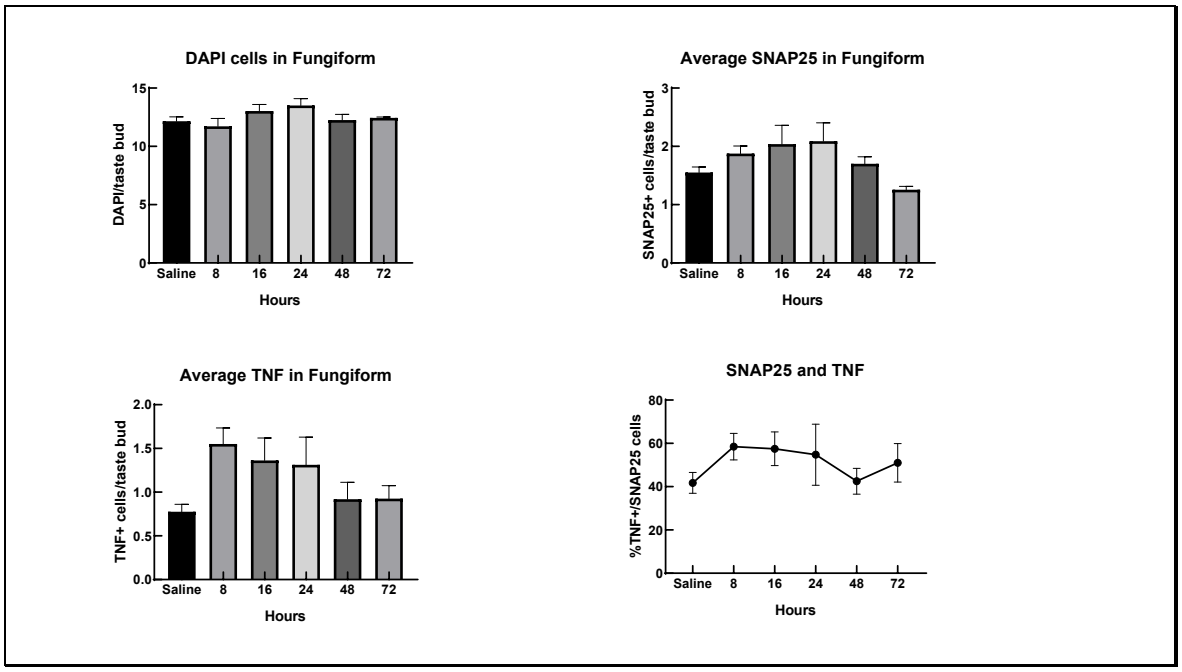


Figure 5: Type III cells in fungiform taste buds. From top left to right: A) The bar graph illustrates the total number of cells per taste bud labeled with DAPI remained constant across 72 hours. B) The mean number of PLCβ2 labeled cells per taste bud in mice over a period of 72 hours. **Bottom left to right:** C) Bar graph indicates the mean number of TNF-α positive cells per taste bud in mice after CYP was administered which showed no difference in response among its groups. D) The line graph illustrates the mean % TNF-α positive cells co-labeled with SNAP-25 in mice. This peaked ($P > 0.05$) at 8 hours after administered CYP (n/group=4).

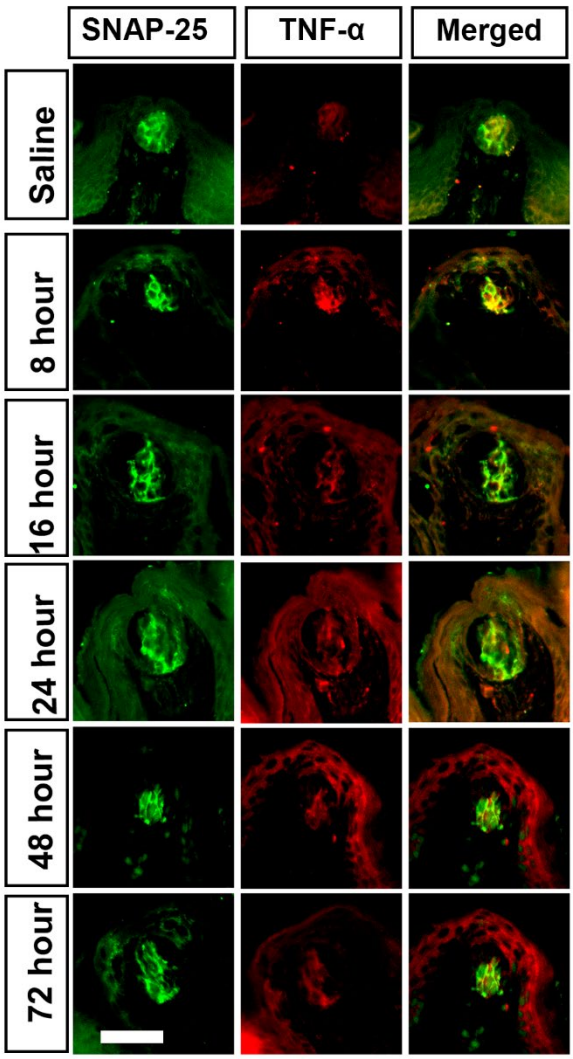
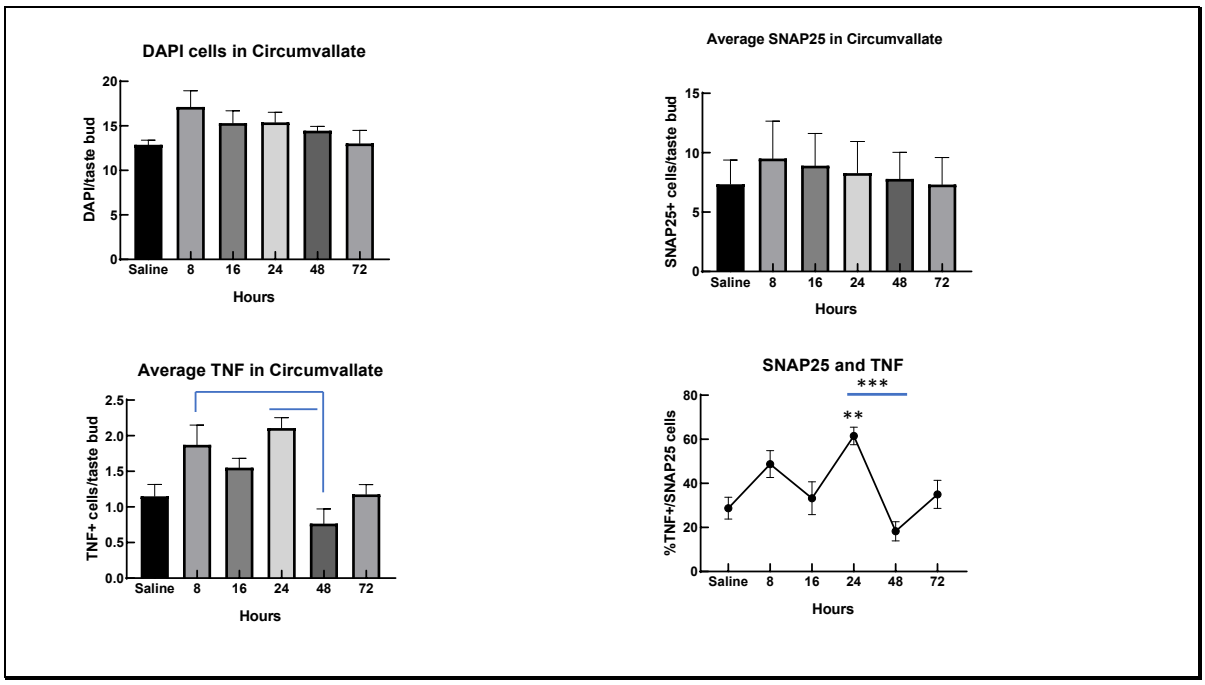


Figure 6. Visualization of Type III cells in fungiform taste buds from mice at 0, 8, 16, 24, 48, and 72 hours post CYP injection. Columns A, B, C show isolated taste buds labeled with SNAP-25 (Green), TNF-α (Red), and cells co-labeled with TNF-α and SNAP-25, respectively. TNF-α expression does not show any significant difference among each of the groups post-CYP injection. The graphical data show a curve at 8 hours post CYP-injection which flattens at 72 hours (n/group=4; scale bar = 20μm).



P<0.01**

P*<0.001**

Figure 7: Type III cells in circumvallate taste buds. From top left to right: A) The bar graph illustrates the total number of cells per taste bud labeled in DAPI remaining constant across 72 hours. B) Total SNAP25 labeled cells per taste bud in mice over a period of 72 hours. **Bottom left to right:** C) Bar graph indicates the mean number of TNF-α positive cells per taste bud in mice after CYP was administered showed peak responses at 8 and 24 hours compared to 48 hours post CYP-injection in mice. D) The line graph illustrates the mean % TNF-α positive cells co-labeled with SNAP25, peaked at 24 hours post CYP-injection in mice after administered CYP (n/group=4).

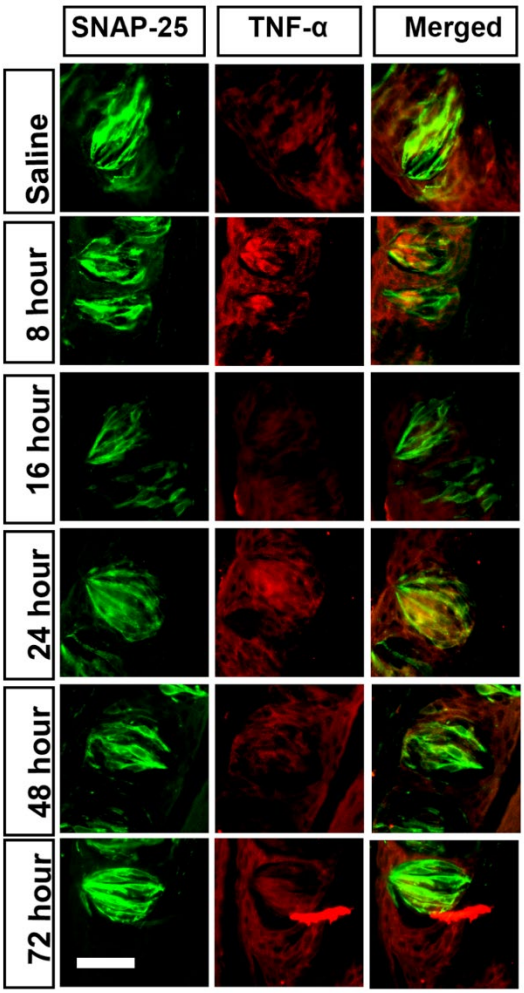
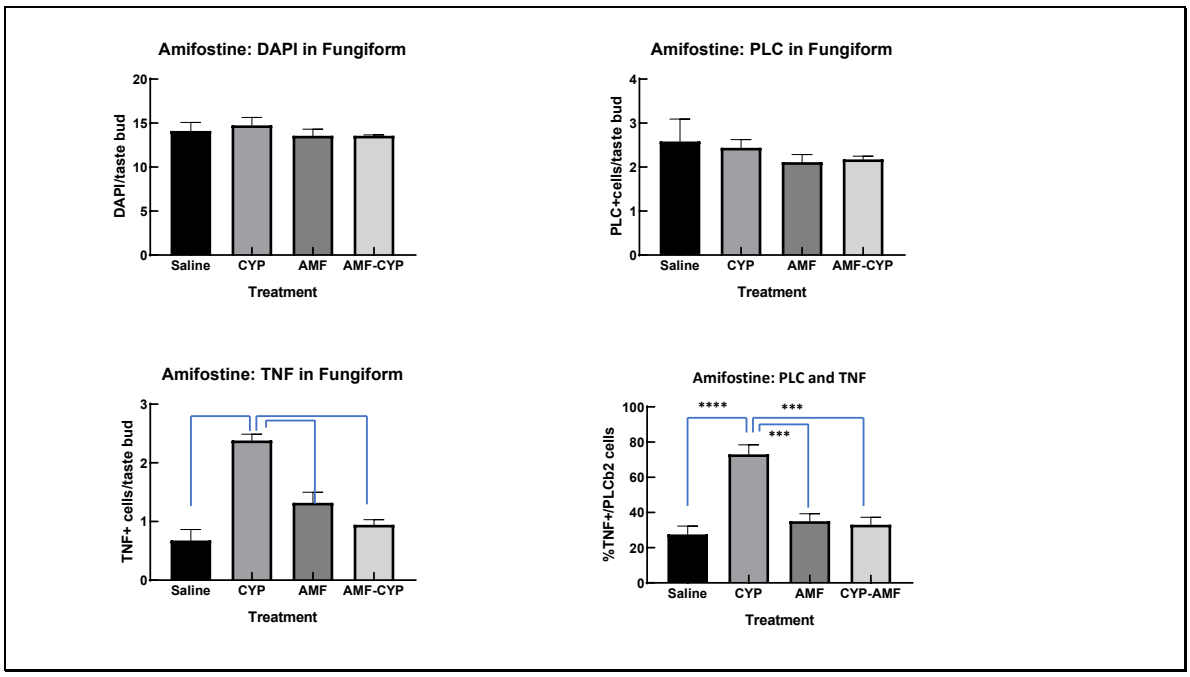


Figure 8. Visualization of Type III cells in circumvallate taste buds from mice at 0, 8, 16, 24, 48, and 72 hours post CYP injection. Columns A, B, C show isolated taste buds labeled with TNF-α (Red), SNAP-25 (Green), and cells co-labeled with TNF-α and SNAP-25, respectively. TNF-α expression peaks at 24 hours post- injection. The graphical data show a peak inflammatory response at 24 hr (n/group=4; scale bar = 20μm).



P**<0.0001**

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Figure 9: Type II cells in fungiform taste buds in different CYP and AMF treatment groups. From top left to right: A) The bar graph illustrates total DAPI labeled cells per taste bud in mice administered saline, CYP, AMF and AMF-CYP remained constant across groups. B) Mean number of PLCβ2 labeled cells per taste bud in saline, CYP, AMF and AMF-CYP mice remained constant across treatment groups. **Bottom left to right:** C) Bar graphs show the mean number of TNF-α positive cells per taste bud in mice after CYP was administered showed peak responses than saline, AMF and AMF-CYP groups. D) The mean % TNF-α positive cells co-labeled with PLCβ2 show significantly higher responses in CYP injected groups compared to saline, AMF and AMF-CYP groups (n/group =4)

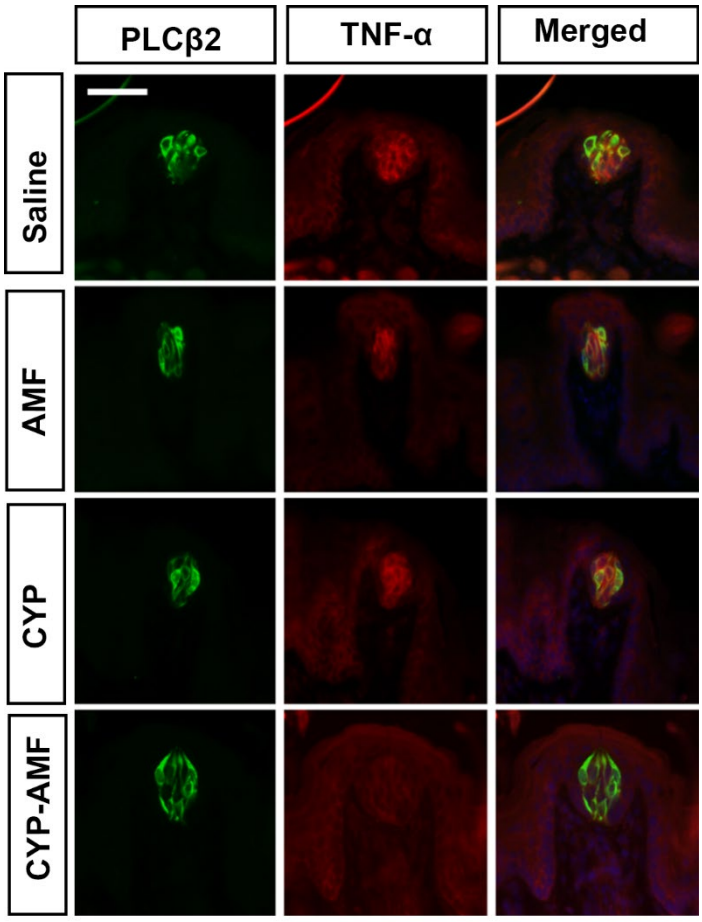
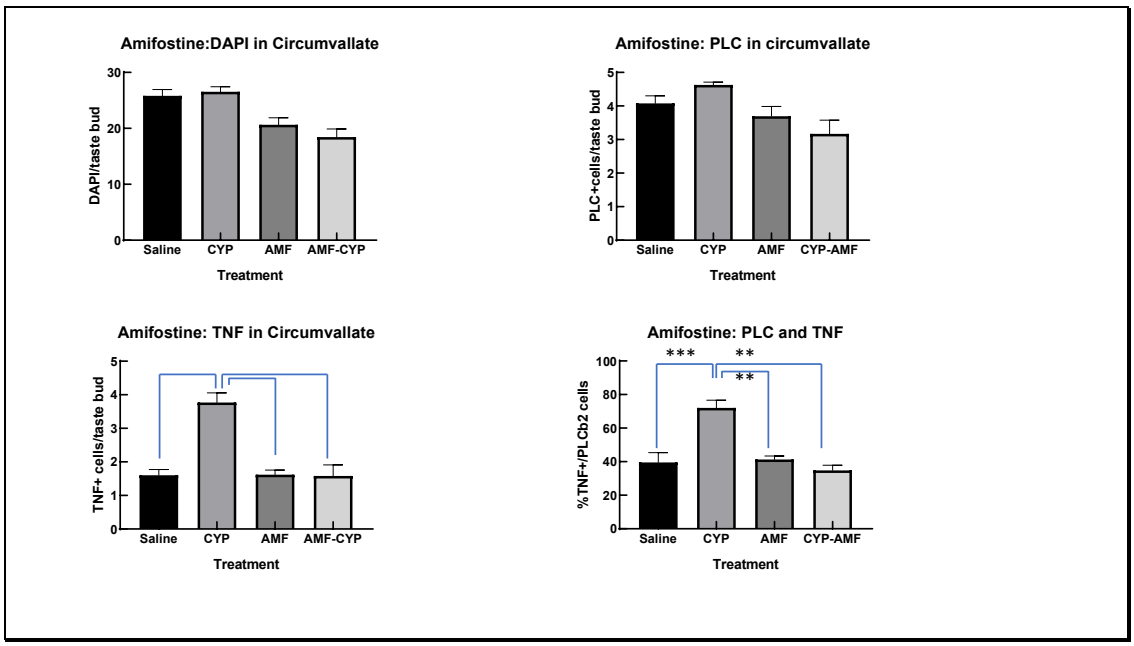


Figure 10. Immunohistochemical assays showing Type II cells in fungiform taste buds from a saline injected mouse, an AMF treated mouse, a CYP treated mouse and an AMF pretreated-CYP mouse. Columns A, B, and C show isolated taste buds labeled with PLCβ2 (Green), TNF-α (Red) and co-label of TNF-α and PLCβ2, respectively. Graphs show less TNF-α expression in AMI pretreated-CYP mice than in CYP-treated mice at 24 hr post injection (n/group=4; scale bar = 20μm).



P*<0.001**

P<0.01**

Figure 11: Type II cells in circumvallate taste buds in different CYP and AMF treatment groups. From top left to right: A) The bar graph illustrates total DAPI labeled cells per taste bud administered saline, CYP, AMF and AMF-CYP mice remained constant across groups. B) Mean number of PLCβ2 labeled cells per taste bud in saline, CYP, AMF and AMF-CYP mice remained constant across treatment groups. **Bottom left to right:** C) Bar graphs show the number of TNF-α positive cells per taste bud in mice after administered with CYP showed peak response compared to AMF and AMF-CYP groups. D) Mean % TNF-α positive cells co-labeled with PLCβ2 was significantly higher in the CYP injected group compared to AMF and AMF-CYP groups (n/group=4)

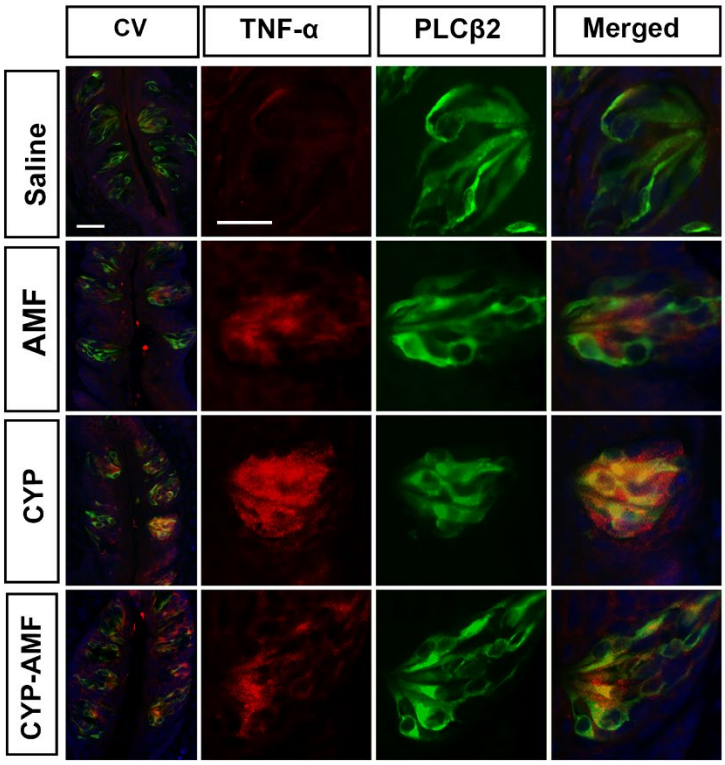


Figure 12. Immunohistochemical assays showing Type II cells in circumvallate taste buds from a saline injected mouse, an AMF treated mouse, a CYP treated mouse and an AMF pretreated-CYP mouse. Column A shows the circumvallate trench with TNF-α labeling (Red), PLCβ2 labeling (Green) and DAPI nuclear stain (Blue) co-labeled. Columns B, C, and D show isolated taste buds labeled with TNF-α, PLCβ2, and co-label of TNF-α and PLCβ2, respectively. Graphs show less TNF-α expression in AMI pretreated-CYP mice than in CYP-treated mice at 24 hr post injection (n/group=4; scale bars = 20μm).

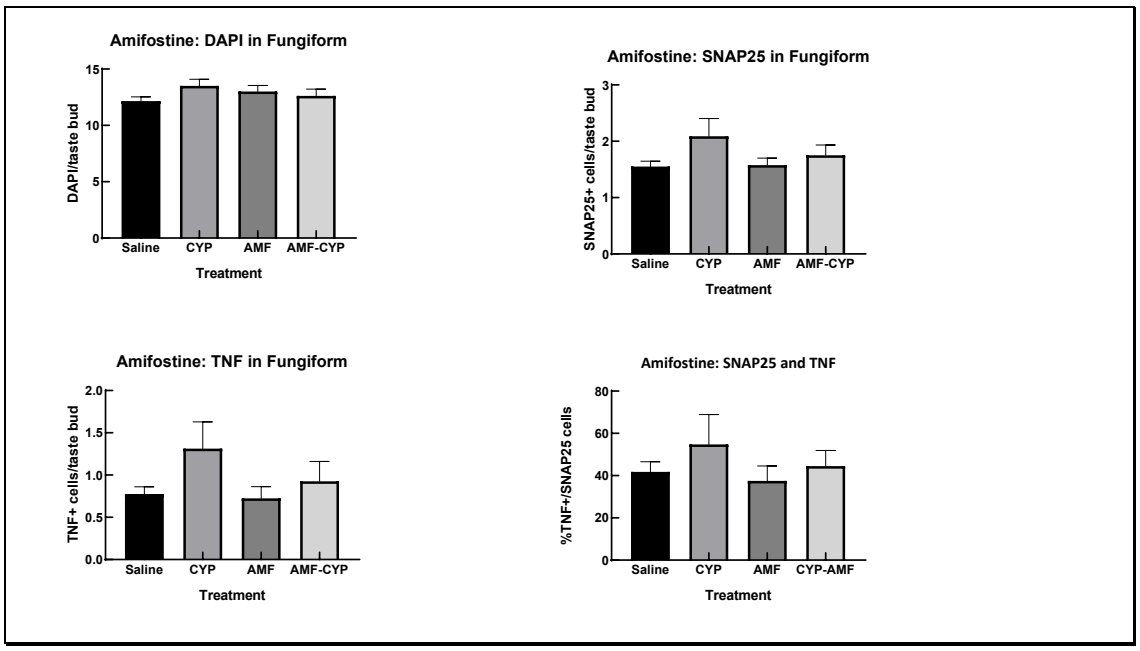


Figure 13: Type III cells in fungiform taste buds in different CYP and AMF treatment groups. From top left to right: A) The bar graph illustrates the total number of DAPI labeled cells per taste bud in mice administered saline, CYP, AMF and AMF-CYP remained constant across groups. B) The mean number of SNAP-25 labeled cells per taste bud in mice administered saline, CYP, AMF, or AMF-CYP remained constant across treatment groups. **Bottom left to right:** C) Bar graphs showing significantly higher number of TNF-α positive cells per taste bud in mice administered CYP compared to AMF and AMF-CYP groups. D) Mean % TNF-α positive cells co-labeled with SNAP-25 show a significantly higher response in the CYP injected group compared to saline, AMF and AMF-CYP groups (n/group=4)

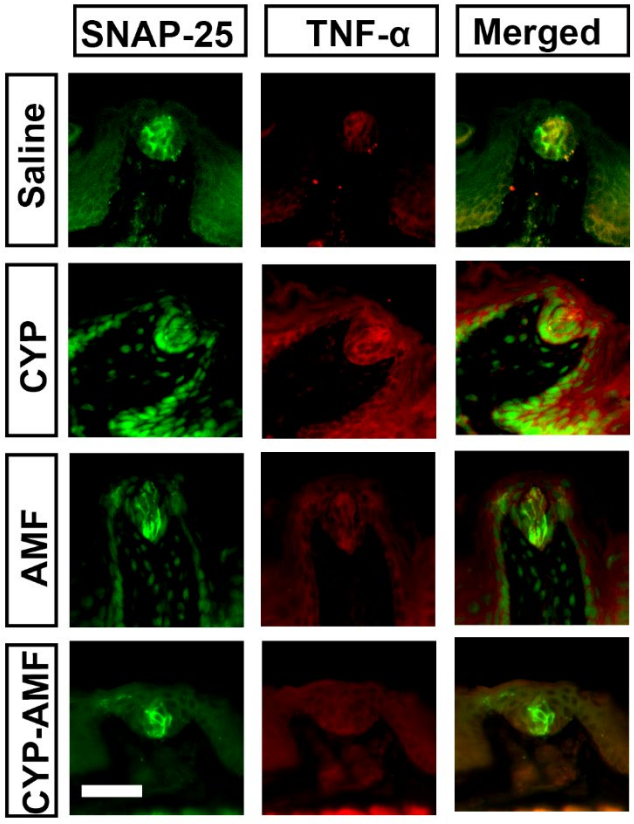
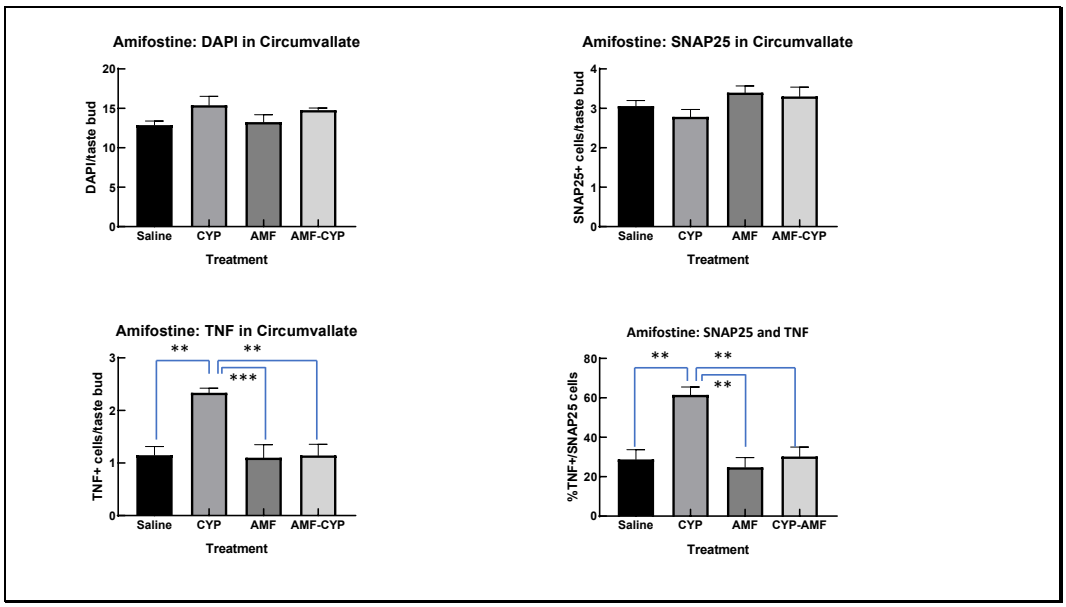


Figure 14. Immunohistochemical assays showing Type III cells in fungiform taste buds from a saline injected mouse, an AMF treated mouse, a CYP treated mouse and an AMF pretreated-CYP mouse. Columns A, B, and C show isolated taste buds labeled with SNAP-25 (Green), TNF-α (Red) and co-label of TNF-α and SNAP-25, respectively. Graphs show less TNF-α expression in AMI pretreated-CYP mice than in CYP-treated mice at 24 hr post injection (n/group=4; scale bar = 20μm).



P*<0.001**

P<0.01**

Figure 15: Type III cells in circumvallate taste buds in different CYP and AMF treatment groups. From top left to right: A) The bar graph illustrates total DAPI labeled cells per taste bud in mice administered saline, CYP, AMF and AMF-CYP remained constant across groups. B) The mean number of SNAP-25 labeled cells per taste bud in saline, CYP, AMF and AMF-CYP mice remained constant across treatment groups. **Bottom left to right:** C) Bar graphs show the number of TNF-α positive cells per taste bud in mice after administered with CYP showed peak response compared to saline, AMF and AMF-CYP groups. D) Mean % TNF-α positive cells co-labeled with SNAP-25 in mice was significantly higher in CYP injected groups compared to saline, AMF and AMF-CYP groups (n/group=4)

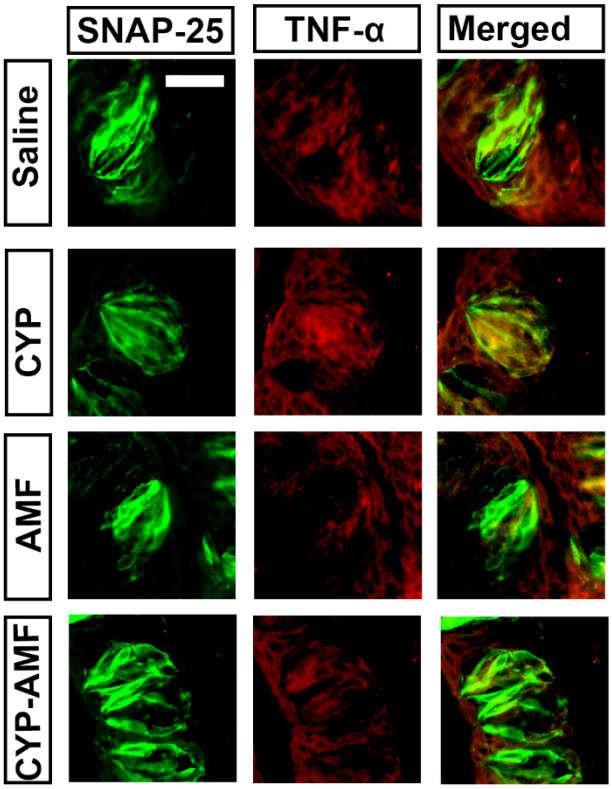


Figure 16. Immunohistochemical assays showing Type III cells in circumvallate taste buds from a saline injected mouse, an AMF treated mouse, a CYP treated mouse and an AMF pretreated-CYP mouse. Columns A, B, and C show isolated taste buds labeled with SNAP-25 (Green), TNF-α (Red), and co-label of TNF-α and PLCβ2, respectively. Graphs show less TNF-α expression in AMI pretreated-CYP mice than in CYP-treated mice at 24 hr post injection (n/group=4; scale bar = 20μm)

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