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The Effects of a Prolonged Administration of Amifostine on the Taste System

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The Effects of a Prolonged Administration of Amifostine on the Taste System

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Abstract

Cyclophosphamide (CYP) is a nonspecific chemotherapeutic drug that negatively disrupts the mammalian taste system. In order to alleviate this disruption, amifostine (AMF), a protective agent, can be administered prior to the introduction of CYP. This study aimed to test for the relative safety of AMF in prolonged exposure with fractionated doses of CYP, which has not been previously conducted in taste epithelium. These effects were studied using selected four and ten-day post-injection time points, where the most significant decrease in taste cell detection thresholds and cell populations had been established in previous studies. One treatment group was injected with a dose of AMF each day over a course of five days and one group was injected with a dose of AMF and CYP each day over a course of five days. Taste cell populations were tracked and compared to control groups using several immunohistochemical markers: Ki67 tagged proliferative cells, PLC β 2 labeled Type II taste cells, and SNAP25 labeled Type III taste cells. Results suggest that there is no significant difference between groups receiving prolonged doses of AMF compared to saline control. In addition, groups receiving AMF prior to fractionated doses of CYP showed significant protection of basal and mature taste cell populations in the circumvallate papillae of the tongue. Information that indicates no change in taste cell populations could elect to be paired with a dose-dense treatment regimen of chemotherapy, with no added toxicity as a byproduct of administration.

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Introduction

Chemotherapy is a common treatment for cancer. However, chemotherapy agents have been under scrutiny for the negative side effects produced in patients. These effects are noticed in a variety of ways, but especially in the form of taste. In a recent study comprised of 387 participants, most reported taste changes immediately following treatment, with 59% of patients continuing to experience taste changes consistently or intermittently throughout the duration of chemotherapy until about four months post-treatment (Bernhardson, Tishelman & Rutqvist, 2007). In this same study, most reported taste changes were associated with altered perception of salty and sweet. This has a significant effect on a patient's quality of life, and more specifically the nutritional aspect of health, as it has been estimated that a substantial number of patients may lose between 10% and 25% of their body weight due to reduced food intake because of nausea, vomiting, and loss of taste preference (Holmes, 1993).

As such, there has been a tremendous need for study in this realm of disease management. Identifying a molecular cause to this incidence is crucial in developing strategies to overcome or ameliorate adverse side effects of chemotherapy, such as loss of taste acuity.

Background of the Taste System

Taste primarily drives our ingestive behavior, food selection, and pairs with olfaction to provide the flavors that we know and love. In addition, the ability to taste functions as a protective mechanism to keep us from ingesting harmful substances. The mammalian taste system is, for the most part, isolated to the lingual region, with some 2000-5000 taste cells housed in three different types of papillae located throughout the epithelial surface of the tongue, which is the primary organ of the gustatory system. Afferent axons synapse with receptor cells

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at the base of the taste bud and convey chemosensory information to the brain, where our perception of taste is realized (Barlow, 2015).

Papillae. Fungiform papillae are located on the anterior two-thirds of the tongue and contain one or two taste buds per papilla. They are innervated by the chorda tympani branch of the facial nerve (cranial nerve VII). The foliate papillae are located bilaterally on the tongue, more posterior to the fungiform, and are innervated partially by the chorda tympani and partially by the glossopharyngeal nerve (cranial nerve IX). Circumvallate papillae are localized to the posterior region of the tongue and appear trench-like under a microscope. These are also innervated by the glossopharyngeal nerve. Each taste bud has several types of cells: supporting cells that contain microvilli, sensory receptor cells which are involved in sensory transduction, and basal cells which later differentiate into receptor cell types (Barlow, 2015; Perea-Martinez, Nagai & Chaudhari, 2013).

Taste Buds. Taste buds are comprised of about 50-100 cells, and taste sensory cells belong to three morphological types: Type I, Type II, and Type III (Finger & Simon, 2000; Barlow, 2015). Each cell type detects a specified subset of tastes (salt, sweet, sour, bitter, and umami) that are structurally related (Finger & Simon, 2000; Perea-Martinez et al., 2013).

Type I cells. Type I cells make up about 50% of the cell population present in taste buds. They contain glial-like processes that wrap around Type II and Type III cells and are difficult to quantify due to their morphological appearance (Bartel et al., 2006). Due to their protein expression profile, they are thought to be involved in the clearing and reuptake of neurotransmitters (Bartel et al., 2006).

Type II cells. Type II cells are considered the principal taste receptors; they make up about 30-35% of the total cell population in a taste bud. Type II cells detect sweet,

bitter, and umami, and mediate a G-protein coupled receptor signal transduction cascade which involves a component of phospholipase C, PLC β 2. PLC β 2 catalyzes the hydrolysis of the molecule PIP₂ into its substituent components IP₃ and DAG. IP₃ triggers the release of internal Ca²⁺ stores from the endoplasmic reticulum, which depolarizes the membrane and leads to neuronal signaling via gustatory nerve fibers (Miyoshi et al., 2001; Barlow, 2015).

Type III cells. Type III cells are the only cell type in the taste bud to make classic synapses on sensory nerve fibers and express SNAP25, a SNARE protein found in synaptic vesicles (Chaudhari, 2014; Clapp, Medler, Damak, Margolskee & Kinnamon, 2006). Type III cells make up about 15% of the population of cells within a taste bud (Barlow, 2015).

Taste Cell Replacement Cycle. Taste cells are continuously turned over and replaced due to age, just like other somatic cells in the body. This natural turnover of cells can be divided into two sub-populations, where the half-life falls between 8 and 12 days for most cells present, with a small fraction of cells living for as long as 24 days (Perea-Martinez et al., 2013). More specifically, Type I cells have a half-life of 8 and 24 days, Type II cells have a half-life of about 8 days, and Type III cells have a half-life of approximately 22 days (Perea-Martinez et al., 2013). It has been hypothesized that all cell types naturally undergo apoptosis and are replaced by populations unique to taste cell type in the adjacent basement membrane, which may differentiate according to transcription factor gradients approximately 2-3 days after relocating to the taste bud (Finger, 2005; Perea-Martinez et al., 2013).

Immunohistochemistry in detecting taste cell populations. To track cell populations, it is customary to use immunohistochemical markers such as Ki67, PLC β 2, and SNAP25. Each of

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these antibody markers labels a certain type of cellular activity that is occurring, such as proliferation or signal transduction. This enables visualization of cell and protein distribution in cell populations under examination. This quantity can be compared to the overall cell population present, which is typically labeled with a counterstain to nucleic acid and is reported as an index.

In the taste system, Ki67 is used as an antibody for proliferative cells. Ki67 is a protein that is present during all active phases of the cell cycle, where it can be localized to the surface of chromosomes (Scholzen & Gerdes, 2000). Thus, a positively-labeled Ki67 population is associated with actively proliferating cells. The PLC β 2 antibody labels its corresponding G-protein that is involved in a secondary messenger cascade for Type II taste transduction. Therefore, this antibody is indicative of Type II cell populations within taste buds (DeFazio et al., 2006). SNAP25 tags a SNARE protein located in synaptic vesicles, and for the purpose of the taste system is used as a proxy for the prevalence of Type III cells since they are the only cell type in the taste bud with a classic synapse (Clapp et al., 2006). Immunohistochemical markers are useful in detecting altered cell populations due to treatment with drugs such as those involved in chemotherapy.

Cyclophosphamide

Cyclophosphamide (CYP) is a useful alkylating agent, most commonly administered in a chemotherapy cocktail, that is characterized by its ability to combat rapid and uncontrolled cell division. It is most commonly used to treat lymphomas, leukemia, and ovarian cancer. CYP is delivered as a pro-drug and is inactive in the body until it passes through the liver and the cytochrome P450 enzyme converts the chemotherapy agent into its active form (Cohen & Jao, 1970). A hydrolytic breakdown of the compound yields phosphoramidate mustard and acrolein as byproducts (Fleer & Brendel, 1982). Phosphoramidate mustard induces significant DNA damage

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through open DNA strand cross-linking while acrolein induces DNA single strand breaks. This ultimately leads to the disruption of DNA synthesis and as a result, the interruption of cell replication and subsequent apoptosis (Crook, Souhami & McLean, 1986). CYP has a half-life of about 6 hours, with a very small amount excreted intact in urine, indicating high uptake in body tissue (Bagley, Bostick & DeVita, 1973).

CYP & taste. While CYP is an effective cancer treatment, it is also a nonspecific agent, as it will initiate and program cell death in normally dividing somatic cells. This is evident in its induction of taste cell disruption. In a study by Mukherjee and Delay in 2011, it was reported that a single 75 mg/kg dose of CYP causes phase shifts in the umami taste thresholds in mice at 4 and 10-days post-injection. This was further characterized by a decrease in the cell populations present in fungiform papillae immediately after injection due to the cytotoxicity of CYP on cells and a decrease in circumvallate taste bud cells about 8-12 days following treatment, which was attributed to an alteration in the replacement cycle of taste cells (Mukherjee & Delay, 2011; Mukherjee, Carroll, Spees & Delay, 2013). This evidence suggests a basis for the reported decrease in taste perception in chemotherapy patients.

Source of replacement cells in taste. Cells which are located in the basal epithelial layer of taste papillae structures are involved in the natural taste cell replacement cycle (Beidler & Smallman, 1965; Delay et al., 1986). This basal progenitor population divides asymmetrically, yielding a stem cell and a transit amplifying cell (Farbman, 1980). Transit amplifying cells eventually become taste sensory cells as they migrate into the taste bud to replenish naturally aging cells (Beidler & Smallman, 1965; Farbman, 1980; Delay et al., 1986). It is speculated in studies involving chemotherapy and taste that the disruption of this progenitor pool ultimately disrupts the replenishment of taste cell populations.

Similar Studies with Irradiation & Taste Loss

Nguyen, Reyland and Barlow (2012) sought to identify the cellular mechanisms underlying taste loss in patients receiving radiotherapy. Researchers found that irradiation-triggered taste dysfunction was a result of the disruption of basal keratinocyte progenitors that replenish taste buds as they die off naturally every 10-14 days (Nguyen et al., 2012). As a result of an increase in apoptotic incidence of progenitor cells in the basement membrane, taste buds were indirectly affected by irradiation, as the recovery of Type II and Type III cells became delayed (Nguyen et al., 2012). This further upholds the previous theory proposed by Mukherjee and Delay (2011): taste acuity is disrupted due to an alteration of the taste cell replacement cycle.

In addition, evidence of this phenomenon is present in a clinical setting, where patients receiving chemotherapy reported less acuity of sweet, umami, and salty tastes, and more heightened perception of bitter and sour (Carson & Gormican, 1977). As a direct result of an altered taste-replacement cycle, a change in taste perception will be noticed until chemotherapy treatment is brought to an end (Gamper et al., 2012). However, there are products available that seek to minimize the loss of this type of disruption in cell population, such as amifostine.

Amifostine

Amifostine (AMF) is an FDA-approved compound administered with the intent of protecting against the death of healthy cells as a result of chemotherapy and radiation treatment in cancer patients. AMF protects somatic cells by scavenging free oxygen radicals, and is shown to be effective mechanistically due to its higher pH and vascular permeability in somatic tissue as compared to cancerous tissue (Andreassen, Grau & Lindegaard, 2003; Culy & Spencer, 2001). In addition, healthy cells have a higher concentration of the enzyme alkaline phosphatase, which is required to metabolize the drug into its active component, WR-1065 (Andreassen et al., 2003;

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Culy & Spencer, 2001). WR-1065 stabilizes genes involved in DNA repair and regulating the cell cycle (Grochova & Smardova, 2007). This type of protective activity is essential for minimizing the adverse side effects seen in patients receiving chemotherapy treatment such as CYP.

The protective effects of AMF on the taste epithelium in the presence of CYP may have medically beneficial results. Before becoming widely available with treatment in 1996, AMF was shown to have a defensive quality over hematopoietic progenitor cells from chemotherapeutics like CYP (List, Heaton, Glinsmann-Gibson & Capizzi, 1996). Later, a number of clinical studies were carried out in which AMF was shown to prevent chemotherapy-induced cytotoxicity of cells (Culy & Spencer, 2001). Phase II and III clinical trials conducted near to the turn of the century continued to show this hallmark minimization of side effects, such as less immune suppression, reduced hematological toxicity, and lessened incidence of neutropenia in chemotherapy treatments like CYP and cisplatin (Aviles et al., 1997; Kemp et al., 1996). Important in these findings is the time period in which AMF is active. With a short half-life ranging between one and eight minutes when administered subcutaneously, only a small percentage of the drug is excreted in urine, indicating that it has a significant uptake in body tissues (van der Vijgh & Korst, 1996). In accordance with these findings, it has become common to administer AMF within the time frame of a half hour to an hour before administering chemotherapy drugs, for maximal antitumor and protective effect (Korst, Boven, van der Sterre, Fichtinger-Schepman, & van der Vijgh, 1998).

AMF & taste. Prior to a 2013 study conducted by Mukherjee and colleagues, the efficacy of AMF had not been tested in chemotherapy-induced taste dysfunction. In the aforementioned study, AMF was administered subcutaneously in a 100-mg/kg dose prior to a 75-

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mg/kg intraperitoneal injection of CYP in mice. Results from this study suggest that AMF protects against CYP-induced inhibition of cell proliferation; there was an improved morphological status of certain cell structures involved in taste and partial protection of taste cells housed within taste buds (Mukherjee et al., 2013). This finding suggests the preservation of taste progenitor cells in the basal epithelial layer where immature cells are produced to replace aging, differentiated taste cell types located within taste buds (Miura & Barlow, 2010). In addition, AMF pretreatment improved taste sensitivity in comparison to treatment with only CYP, devoid of AMF (Mukherjee et al., 2013). From this, it can be deduced that combining AMF with CYP sustains the normal replacement of taste cells.

Chemotherapy Regimens

Chemotherapy can be administered a number of ways to a patient depending on the type of cancer present, and can be given on a weekly or monthly basis. Dose-dense administration of chemotherapy, or dose fractionation, can best be described by reducing the time interval between administration of the drug without increasing the dose. This type of treatment is administered with the intent of interrupting the rapid growth phase of tumor cells (Simon & Norton, 2006; Kummel, Rezai, Kimmig & Schmid, 2007). Essentially, increasing the frequency of treatment serves to sustain the amount of active drug in the body and act on populations that have been depleted since the last course of treatment. This seeks to decrease the chance of prolonged neoplastic development. There seems to be a bit of a double-edge sword in the administration of chemotherapy agents, as too low of a dose will likely be rendered ineffective, while too high of a dose may lead to myelosuppression and cardiac toxicity (Moore, 2012). Since AMF has not been studied in this way on the taste system, evidence of the protective nature of AMF with fractionated doses of CYP could be crucial in how it is paired with chemotherapy drugs. As

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such, the current investigation was conducted to see if AMF can protect the taste system without detrimental side effects.

Description of Previous Study

A study conducted previously by S. Socia, an undergraduate at University of Vermont and personal communication, tested for possible cell population differences in circumvallate papillae in comparing a treatment group receiving one “moderate dose” of CYP (75 mg/kg) to a treatment group receiving five “fractionated doses” of CYP (15 mg/kg administered each day for five days), modeling dose fractionation of chemotherapy drugs. The last injection day was designated “Day 0” and mice were perfused every two days thereafter until the 16-day time point. Taste cell populations were analyzed with three immunohistochemical markers: Ki67, PLC β 2, and SNAP25 to characterize any morphological differences in taste cell populations as a result of receiving CYP in one large dose or in smaller doses that were administered over a number of days.

This study found evidence to suggest that both the low dose (5*CYP) and high dose of CYP (1*CYP) have significant effects on populations of Type II cell populations over the 16-day time period post-injection as compared to the saline control. More specifically, at day-4 and 10 post-injection, the 5*CYP groups shows an observed decrease in their mean index of PLC β 2-positive cells to total cell population in the circumvallate papillae. In addition, the data suggest that Ki67-positive cell populations are diminished at the 4-day timepoint for both treatment groups (5*CYP and 1*CYP), with the 5*CYP cell population continuing to experience diminished proliferation at day 6 post-injection. Ki67 being a proliferative cell marker, this may suggest that cells of the basal layer that are involved in taste cell replacement may be affected as a result of treatment.

Objectives of Current Study

This research was conducted with the intent to characterize the effect of multiple exposures of AMF on the taste system. It is known that the body's uptake and metabolism of CYP is seen in the form of adverse side effects, such as the loss of appetite, nausea, hair loss, and frailty (Bernhardson et al., 2007). Certainly, reducing these side effects is a paramount quality of designing a better chemotherapy treatment pattern. Potentially, this could be achieved through pre-treatment with AMF. However, it is important to define AMF's mechanism of action and determine if it, like CYP or other common chemotherapy agents, is detrimental to the taste system. This has not been determined prior, despite the extensive amount of work which has been conducted in evaluating AMF in the presence of chemotherapy agents in other body tissues.

In this study, AMF was administered at 100 mg/kg each day over a course of five days or in one, single dose (100 mg/kg), and comparisons were drawn between cell populations. In addition, AMF treatment was paired with one moderate dose (75 mg/kg) or five fractionated doses (15 mg/kg) of CYP in order to test for the maintenance of AMF as a cytoprotective agent modeling dose fractionation chemotherapy treatment. Cell proliferation and changes, if any, in Type II and Type III cell populations were studied using markers of Ki67, PLC β 2, and SNAP25, respectively. These were studied at the 4-day and 10-day post-treatment timepoints as per previous data (S. Socia, personal communication) suggesting the decreased PLC β 2 and Ki67-positive cells present in taste buds of the circumvallate papillae at these time points. This study of taste cell populations is a means of tracking the effects of AMF on normally dividing cells in the taste epithelium as well as in the taste buds themselves.

It was hypothesized that prolonged administration of AMF over a course of five days at 100 mg/kg would not show significant morphological changes from its counterparts in this study,

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where there is another treatment group receiving one dose of AMF (100 mg/kg) and a saline control. In addition, it was hypothesized that AMF would be effective in minimizing the taste cell population lost as a result of five low doses of CYP.

Methods

Subjects

A total of 40 C57Bl/6J mice from Jackson Laboratories in Bar Harbor, Maine were used in this study. Subjects were at least six weeks old and housed in groups of four in cages on a 12-hour light cycle/12-hour dark cycle—the light cycle started at 7 AM. Mice were given a week to acclimate to the room in which they were kept in following arrival at the animal care facilities. The room was kept at 25°C and 60% humidity. The mice were fed with Purina mouse chow and water *ad libitum*. University of Vermont veterinary technicians monitored the mice each day over the course of the study.

Experimental Design

Injections. The subjects were grouped into seven treatment groups: (1) Saline (s): received a saline intraperitoneal (IP) injection each day over a course of five days, (2) 1*CYP: one IP injection of saline each day for four days, with one IP injection of CYP (75 mg/kg) on the fifth day, (3) 5*CYP: one IP injection of CYP (15 mg/kg) each day for five days, (4) 1*AMF: one IP injection of saline each day for four days with one subcutaneous (SC) injection of AMF (100 mg/kg) on the fifth day, (5) 5*AMF: one SC injection of AMF (100 mg/kg) each day for five days, (6) 1AMF*1CYP: four IP injections of saline over the course of four days with one SC injection of AMF (100 mg/kg) 30 minutes prior to one IP injection of CYP (75 mg/kg) on the fifth day, (7) 5AMF*5CYP: one SC injection of AMF (100 mg/kg) administered 30 minutes prior to one IP injection of CYP (15 mg/kg) each day over the course of five days (Table 1).

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These injections were conducted at approximately the same time each day for consistency in analysis of time-dependent tissue in future methods. Four sets of mice were used per designated time point (4-day post-injection and 10-day post-injection).

Table 1: Treatment Groups of Mice. Each treatment group has a mouse for each time point perfused. These injection procedures were carried out in 4 sets of mice. Saline mice receive IP saline injections each day for five days. 1*CYP groups receive one moderate IP dose of CYP on the fifth day of injections. 5*CYP groups receive an IP fractionated dose of CYP each day for five days. All AMF SC injections were delivered at a 100 mg/kg dose.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
<i>Saline (s)</i>	s/s	s/s	s/s	s/s	s/s
<i>1*CYP (C)</i>	s	s	s	s	C
<i>5*CYP (c)</i>	c	c	c	c	c
<i>1*AMF (a)</i>	s/s	s/s	s/s	s/s	A/s
<i>5*AMF (a)</i>	A/s	A/s	A/s	A/s	A/s
<i>1AMF*1CYP (C)</i>	s/s	s/s	s/s	s/s	A/C
<i>5AMF*5CYP (c)</i>	A/c	A/c	A/c	A/c	A/c

Mice injected with CYP required a cage change 24 hours after injection, as the phosphoramidate mustard emitted as a byproduct of CYP metabolism is excreted in urine and may be toxic if it dries and turns to powder. The last day of injections were designated as day 0. Following this, half of the mice in the study were perfused at 4-day post-injection, and half were perfused 10-day post-injection. These time points were selected considering findings from a previous study (S. Socia, personal communication), in which the largest disturbance of taste cell populations were observed at these times post-injection of CYP. Perfusions were conducted using 0.1 M PBS plus heparin and 4% paraformaldehyde in 0.1M PBS. Three hours after the perfusion the tongues were transferred to a 30% sucrose solution to cryoprotect the tissues.

Blocking. Twenty-four hours after perfusion, the tongue tissue was blocked into three sections according to regions of papillae, anterior to posterior: fungiform, foliate, and

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circumvallate segments. They were placed in Tissue-Tek molds and stored in a freezer at a temperature of -80°C .

Sectioning. The circumvallate papillae from each sample was sectioned at a $12\ \mu\text{m}$ section thickness using a cryostat. Slides were stored at -20°C .

Immunohistochemistry. Immunohistochemical staining is used to better understand the physiological change and distribution of types of taste cells present on tongue sections. Several markers are utilized to tag these, the markers used include: PLC β 2, which is found in Type II cells, SNAP-25 found in Type III cells, and Ki67 for actively dividing cells. PLC β 2 was used at a 1:1000 dilution (H-255, Santa Cruz Biotechnology), SNAP-25 at a 1:5000 dilution (S5187, SIGMA), and Ki67 (SP6, Thermo Scientific) at a 1:200 dilution. Slides were allowed to incubate and stored overnight at 4°C . The secondary antibody used was Alexa 546 goat anti-rabbit at a dilution of 1:1000 with incubation at room temperature for 2 hours. Slides were counterstained with Sytox green (S7020, Molecular Probes) as a nuclear marker.

Image acquisition and counting. Images were captured using a Nikon Eclipse E600 Scope (Spot RT KE Diagnostic Instruments Inc.). The computer program SPOT (Spot Advanced, Version 4.6) was used to acquire images of the immunohistochemically stained tissue. Following this, the cells were quantified blindly using ImageJ software.

Statistical analysis. Cell populations were analyzed at each time point by quantifying the ratio of positively-labeled cells within specific taste structures by the total number of cells present in each section of tissue sampled according to the nuclear label for Ki67, PLC β 2, and SNAP25.

Data were analyzed by Repeated Measures ANOVA followed by a simple effect test for reported index per treatment group for each timepoint post-treatment. Statistical differences were

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drawn between two major independent variables: treatment group and time point post-treatment. This test was run for each dependent variable, which was the ratio of Ki67, PLC β 2, and SNAP 25 positively-labeled cells to the Sytox green counter-stain. With a 7x2 factorial design, I used a between subjects univariate ANOVA looking for significant effects for drug conditions (7) and days post-injection (2). This was further evaluated by an alpha-corrected post hoc test.

All statistical analysis was conducted using SPSS software (IBM SPSS Statistics, Version 24, IBM Corporation, Chicago, IL). All figures were made using Graph Pad Prism software, Version 7.0 (Graphpad software Inc., La Jolla, CA, USA).

Results

AMF Protects Against Cell Loss in Proliferative Cell Population

A univariate ANOVA was conducted to examine the effect of treatment group and timepoint post-injection on the ratio of positively-labeled Ki67 cells to the total cell population. There was a statistically significant interaction between the effects of treatment group and timepoint post-injection on the ratio, $F(6, 40) = 2.599$, $p = 0.042$. Post hoc comparisons using the Sidak multiple comparisons test indicated that the mean indices for 1*CYP ($M = 0.390$, $SEM = 0.048$) and 5*CYP ($M = 0.437$, $SEM = 0.058$) were significantly different than saline control ($M = 0.824$, $SEM = 0.048$) at the 4-day post-injection timepoint. This incidence is apparent in observed differences between saline control group, 1*CYP, and 5*CYP groups at 4-days post-injection (Figure 1). The observed decrease in the index at 4-days post-injection for the 1*CYP group is in line with previous findings by Mukherjee et al. (2011), in which there was a reported decrease at day-4 post-injection for the group receiving one 75 mg/kg dose of CYP.

Furthermore, there was not a statistically significant difference between the 1*CYP and 5*CYP

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groups at this timepoint, indicating that the chemotherapy regimens affect the proliferating cell population in a similar manner.

In addition, post hoc evaluation revealed that the mean indices for 1*AMF (M = 0.702, SEM = 0.048) and 5*AMF (M = 0.689, SEM = 0.048) did not differ significantly from the saline group (M = 0.824, SEM = 0.048) at 4-day post-injection (Figure 2). At 10-day post-injection, 1*AMF (M = 0.667, SEM = 0.048) and 5*AMF (M = 0.659, SEM = 0.041) also did not differ significantly from the saline group (M = 0.784, SEM = 0.048) (Figure 2).

Post hoc evaluation indicated that groups receiving AMF prior to CYP have significantly different indices than groups solely receiving CYP. At 4-days post-injection 1AMF*1CYP (M = 0.706, SEM = 0.058) significantly differed from 1*CYP (M = 0.390, SEM = 0.058) (Figure 3). In addition, at this timepoint 5AMF*5CYP (M = 0.738, SEM = 0.041) differed significantly from 5*CYP (M = 0.437, SEM = 0.048) (Figure 3). Both 1AMF*1CYP and 5AMF*5CYP did not differ significantly from the saline control, indicating that AMF was able to protect against the disruption of the taste cell progenitor population that occurs when groups only receive CYP treatment.

AMF Protects Against Cell Loss in Type II Taste Sensory Cells

A univariate ANOVA was conducted to examine the effect of treatment group and timepoint post-injection on the ratio of positively-labeled PLC β 2 cells to the total cell population. There was not a statistically significant interaction reported between the effects of treatment group and timepoint post-injection on the ratio, $F(6, 43) = 0.973$, $p = 0.43$. However, further post hoc evaluation revealed that there were statistically significant differences in mean indices on day-4 post-injection for 5*CYP (M = 0.159, SEM = 0.014) and saline (M = 0.270, SEM = 0.010) (Figure 4). In addition, Sidak multiple comparisons post hoc test reported that

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the mean indices for 1*CYP (M = 0.178, SEM = 0.014) and 5*CYP (M = 0.151, SEM = 0.012) differed significantly from saline (M = 0.240, SEM = 0.010) at day-10 post-treatment (Figure 4). The reported decrease of the 1*CYP group at day-10 post-injection is directly in line with the findings of Mukherjee et al. (2011), where the group receiving a 75 mg/kg dose of CYP exhibited a decrease in label positive PLC β 2 compared to total cell population at that same timepoint. It is likely that the original ANOVA analysis was unable to detect these differences in mean indices due to random sampling error, or the small number of indices reported per animal in the CYP treatment groups (n = 2). As revealed in post hoc test, there were significant results obtained when the testing became more stratified and further corrected for multiple comparisons.

Post hoc evaluation indicated that the mean indices for 1*AMF (M = 0.242, SEM = 0.012) and 5*AMF (M = 0.244, SEM = 0.012) did not differ significantly from the saline (M = 0.270, SEM = 0.010) at 4-day post-injection (Figure 5). At 10-day post-injection, 1*AMF (M = 0.238, SEM = 0.012) and 5*AMF (M = 0.221, SEM = 0.012) also did not differ significantly from the saline group (M = 0.240, SEM = 0.010 (Figure 5).

Furthermore, post hoc multiple comparisons tests revealed that groups receiving AMF prior to CYP had a significantly larger mean index than groups only receiving CYP, as indicated by mean (SEM) PLC β 2-positively labeled cells of the CYP compared to the total cell population of the CYP. At day-4 post-injection, 5AMF*5CYP (M = 0.232, SEM = 0.010) differed significantly from 5*CYP (M = 0.159, SEM = 0.014) (Figure 6). At 10-days post-injection, a significant difference was reported between both chemotherapy regimens and their corresponding AMF*CYP treatment groups. The mean index of 1AMF*1CYP (M = 0.219, SEM = 0.010) differed significantly from that of 1*CYP (M = 0.178, SEM = 0.014) (Figure 6).

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In addition, the mean index of 5AMF*5CYP ($M = 0.243$, $SEM = 0.012$) differed significantly from 5*CYP ($M = 0.151$, $SEM = 0.012$) (Figure 6).

AMF Protects Against Cell Loss in Type III Taste Sensory Cells

A univariate ANOVA was conducted to examine the effect of treatment group and timepoint post-injection on the ratio of positively-labeled SNAP25 cells to the total cell population. There was a statistically significant interaction reported between the effects of treatment group and timepoint post-injection on the ratio, $F(6, 34) = 11.708$, $p = 0.001$. Post hoc comparison using the Sidak multiple comparisons test revealed that the 1*CYP ($M = 0.103$, $SEM = 0.005$) differed significantly in its mean index from saline ($M = 0.166$, $SEM = 0.005$) at 4-days post-injection (Figure 7). There were no differences reported in mean indices between the 5*CYP and the saline control group at this same timepoint. This may be attributed to only having counts for a small number of animals at this timepoint.

Further post hoc evaluation revealed that the mean indices for 1*AMF ($M = 0.165$, $SEM = 0.004$) and 5*AMF ($M = 0.159$, $SEM = 0.004$) did not differ significantly from saline ($M = 0.166$, $SEM = 0.005$) at day-4 post-treatment (Figure 8). At day-10 post-injection, 1*AMF ($M = 0.161$, $SEM = 0.004$) and 5*AMF ($M = 0.156$, $SEM = 0.004$) also did not differ significantly from saline ($M = 0.165$, $SEM = 0.005$) (Figure 8).

Type III cell populations were largely protected in groups receiving AMF prior to CYP as compared to groups solely receiving CYP. At day-4 post-treatment, the mean index of 1AMF*1CYP ($M = 0.156$, $SEM = 0.004$) differed significantly from that of 1*CYP ($M = 0.103$, $SEM = 0.005$) (Figure 9). There were no significant differences reported between 1*CYP and 1AMF*1CYP 5*CYP and 5AMF*5CYP groups at day-10 post-injection, as there were no statistically significant decreases in groups receiving CYP at this particular timepoint.

Discussion

In this study, it was hypothesized that there would be no significant difference between saline control groups and animals receiving either one 100 mg/kg dose or a 100 mg/kg dose of AMF each day for five days. It was also hypothesized that groups receiving doses of AMF prior to injection of CYP would show significant improvement from animals solely receiving CYP injections. Using data derived from a previous study carried out by S. Socia, personal communication, and a study by Mukherjee and colleagues (2013) the four and ten-day post-injection time points were selected in studying the morphological effects of one 100 mg/kg dose and five 100 mg/kg doses of AMF on taste cell populations in the circumvallate trench and papillae.

CYP Treatment Causes Disturbance in Proliferative and Mature Taste Cell Populations

The data suggest that there are decreases in cell populations when subjected to one moderate dose of CYP or five fractionated doses of CYP. There is a decrease in Ki67-positively labeled cell populations to the total cell population at 4-days post-injection for both 1*CYP and 5*CYP treatment groups (Figure 1). This indicates that there is a lessened progenitor pool of proliferative cells at this timepoint. A finding such as this may predict the disruption of the natural taste cell replacement cycle, as replenishment of taste bud cell populations is negatively affected. This result is in line with previous findings by Mukherjee et al. (2011) and Nguyen et al. (2012), where disturbances in Ki67-positively labeled cell populations are noticed soon after treatment administration, at day-4 and 2, respectively.

In addition, a decrease in Type II cells, as evidenced by the ratio of PLC β 2-positively labeled cell populations to the total cell population, was reported at day-4 for the 5*CYP group and day-10 post-injection for both 1*CYP and 5*CYP treatment groups (Figure 4). A decrease

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was noted in SNAP25-positively labeled cell populations at day-4 post-injection for the 1*CYP treatment group (Figure 7). It was expected that the 5*CYP group would follow similar index patterns over the time-course as the 1*CYP group. This disturbance in Type III cell populations may be due to a low “n” for this treatment group at this timepoint. These data indicate that there is a disturbance in replenishment of mature taste cell populations following administration of both chemotherapy regimens. This may be due to the alteration of the taste cell replacement cycle.

AMF Does Not Introduce Toxicity to Taste Cell Populations

The data suggest that there is no molecular difference, as described by the index of positively-labeled cell populations to total cell populations, between the control group and groups where AMF is administered at either one single 100 mg/kg dose or five 100 mg/kg doses. This finding corroborates the original hypothesis. With no statistical difference between saline control and both 1*AMF and 5*AMF groups, it appears that AMF does not introduce toxicity to cells at the level of the taste system. This is in comparison to a DNA alkylating agent such as CYP, where decreases are noticed in mature cell populations as little as 4-days post-injection in the fungiform papillae due to initial toxicity, and 8-days post-injection in the circumvallate papillae (Mukherjee & Delay, 2011) (Figures 2, 5 & 8).

Furthermore, with no significant difference occurring between 1*AMF and 5*AMF groups, it appears that there is no added toxicity despite multiple exposures to AMF (Figures 2, 5 & 8). Data that suggest minimal to no toxicity are important for future clinical application, as toxicity studies carried out in animal models not only help to characterize the safety of a drug but also provides a basis for determining a potential dosage in humans (Arome & Chinedu, 2013).

AMF Prevents CYP-Mediated Taste Cell Population Disturbances

A significant difference was observed between groups receiving 1*CYP and 5*CYP, and groups that had AMF injected prior to CYP administration (Figures 3, 6 & 9). This occurrence was evidenced by the number of Ki67-positive cells in the basal lamina of the circumvallate trench on day-4 post-injection (Figure 1). In addition, a significant difference was noted in mature taste cell populations between both groups receiving AMF*CYP and groups solely receiving CYP on day-4 and day-10 post-injection (Figures 6 & 9). This was gleaned from the number of PLC β 2-positive cells compared to total cell population at day-4 and day-10 post-treatment. In reference to Type II cell populations, the 5AMF*CYP group at day-4 post-injection had a significantly larger index than the group receiving five fractionated doses of CYP (Figure 6). In addition, both the 1AMF*1CYP and 5AMF*5CYP groups had significantly greater mean indices than the groups receiving one moderate dose, or five fractionated doses of CYP over several days (Figure 6). In Type III cell populations, the 1AMF*1CYP group had a vastly improved index as compared to the 1*CYP group at day-4 post-injection (Figure 9).

These data indicate that AMF is effective in reducing the incidence of progenitor cell depletion that is typically seen with CYP exposure, as well as maintaining Type II and Type III cell populations in the circumvallate papillae, which is in line with the literature (Mukherjee & Delay, 2013). The evidence suggests that AMF allows the taste cell replacement cycle to remain partially uninterrupted even with exposure to CYP.

Conclusion

The apparent lack of AMF's added toxicity to the body may be attributed to its mechanism of action. AMF is delivered as a prodrug that is metabolized by alkaline phosphatase, which is an enzyme that is of particularly low concentration in neoplastic tissue, but

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is of higher concentration in normal tissue (Kouvaris, Kouloulis & Vlahos, 2007). In addition, healthy somatic cells are of a more neutral pH and have higher vascular permeability as compared to cancerous tissue, two features which further contribute to a greater metabolism of AMF in healthy cells (Kouvaris et al., 2007). Once inside the cell, AMF works to scavenge oxygen free radicals generated as a result of cell-induced death due to chemotherapy, which protects the cellular membrane from damage (Kouvaris et al., 2007). As a result, normal tissues subjected to the active metabolite may have a 100-fold greater concentration than tumorous tissue, which are hypovascular and have low enzyme levels (Yuhas, 1980). As evidenced by the data at hand, introducing AMF prior to treatment with CYP allows mature, non-tumorous taste cells to remain intact and harbor against CYP's ability to wreak havoc at the cellular level.

Taking the data in sum, it appears that AMF is safe to administer over a number of days, or in one, acute injection with no reported differences in cell populations. This type of information pairs well with trends in administration of chemotherapy treatments, where AMF given prior will likely have cytoprotective effects with no added toxicity. Maintenance and even a reported increase in cell populations following damage has been reported with pre-treatment of AMF in patients receiving chemotherapy due to the upregulation of proteins involved in DNA repair, leading to accelerated recovery (Rubin, Drab, Kang, Baumann & Blazek, 1996). This evidence is promising in maintaining the ability to taste even through chemotherapy treatments.

Future Directions

The data gleaned from this study illustrate important trends in taste cell depletion induced as a result of chemotherapy treatment. Using immunohistochemical staining, it was evident that decreases were especially evident in the proliferative cell population, as well as mature taste cell populations as a result of an altered taste cell replacement cycle. Immunohistochemistry

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methods give a glimpse into one part of what could be a broader picture. Therefore, I believe it would be interesting to conduct a few more tests to ascertain the level of progenitor death, such as through a TUNEL assay, which tags the ends of nicked DNA strands during apoptosis. I also think it may be interesting to conduct BrdU labeling, which incorporates a thymidine derivate during the synthesis stage of the cell cycle and allows newly born cells to be visualized. Certainly, over a time-course study, replenishment of mature taste cell populations within the taste buds from the basal progenitor pool could be visualized. These tests would further corroborate proposed findings from the current study.

In addition, these data have important clinical applications. Results suggest that AMF is successful in protecting against depletion of cell populations with exposure to chemotherapy drugs like CYP, especially in treatment groups modeling dose fractionation, which is a common treatment regimen in humans. This method of administration seems promising for potential improved outcomes in clinical administration.

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Appendix

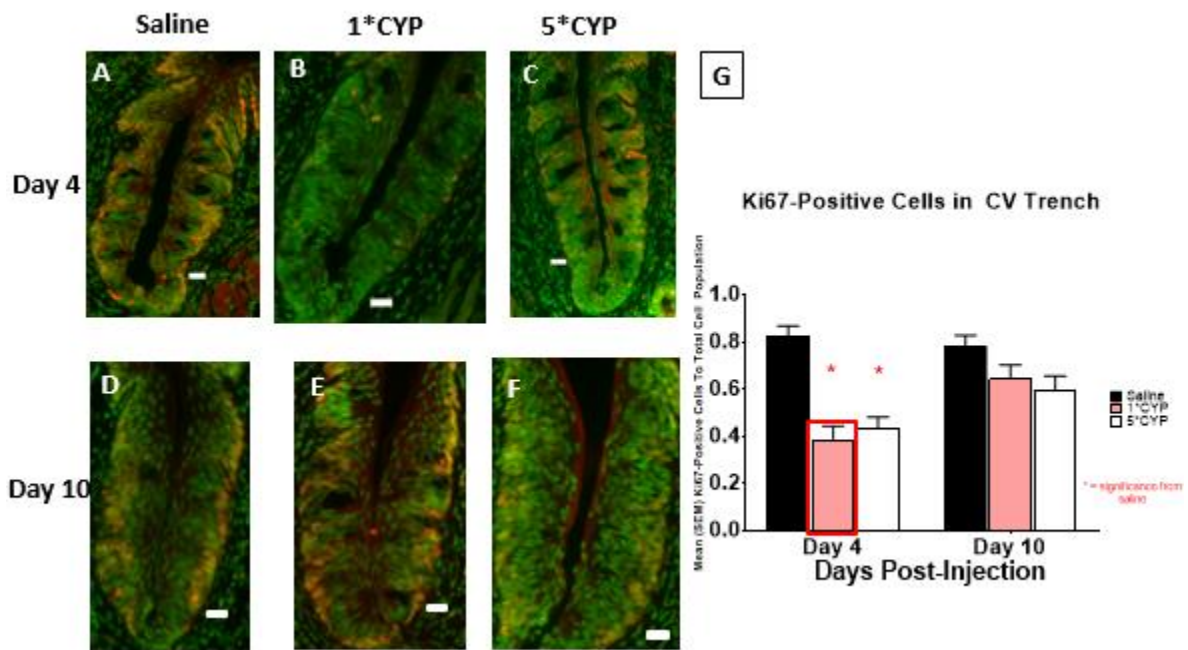


Figure 1: Mean (SEM) of Ki67-positive cells to total cell population in the circumvallate trench for CYP groups. (A-C) Ki67-positive cells (red) in one side of the circumvallate trench at 4-days post-injection for saline, 1*CYP, and 5*CYP (bar = 20 μ m). (D-F) Ki67-positive cells (red) at 10-days post-injection (bar = 20 μ m). (G) Mean (SEM) Ki67-positive cells to total basal cell population in circumvallate trench for each drug condition at day-4 and day-10 post-injection ($p < 0.05$).

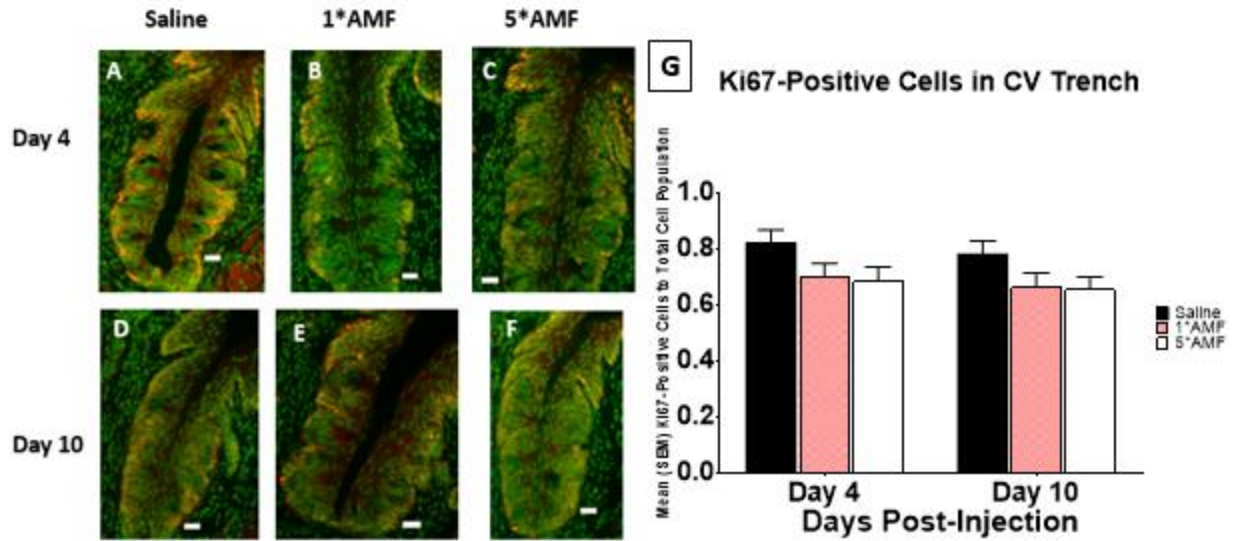


Figure 2: Mean (SEM) of Ki67-positive cells to total cell population in the basal layer of the CV trench for AMF groups. (A-C) Ki67-positive cells (red) in one side of the circumvallate trench at 4-days post-injection for saline, 1*AMF, and 5*AMF. (D-F) Ki67-positive cells at 10-days post-injection for each of the treatment groups (bar = 20 μ m). (G) Mean (SEM) Ki67-positive cells to total cell population in circumvallate trench for each drug condition at day-4 and day-10 post-injection.

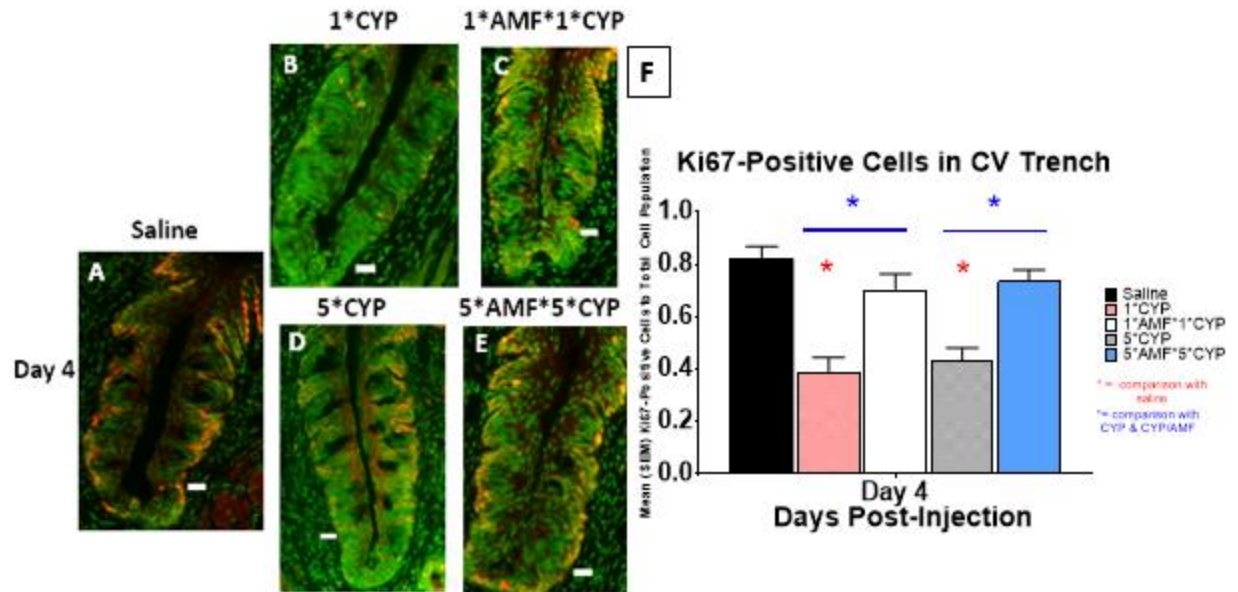


Figure 3: Mean (SEM) Ki67-positive cells to total cell population in CV trench for AMF*CYP groups. (A-E) Ki67-positive cells (red) at 4-days post-injection for saline, 1*CYP, 1AMF*1CYP, 5*CYP, & 5AMF*5CYP (bar = 20 μ m). (F) Mean ratio (SEM) of Ki67-positive cells to total cell population in basal layer of the circumvallate trench

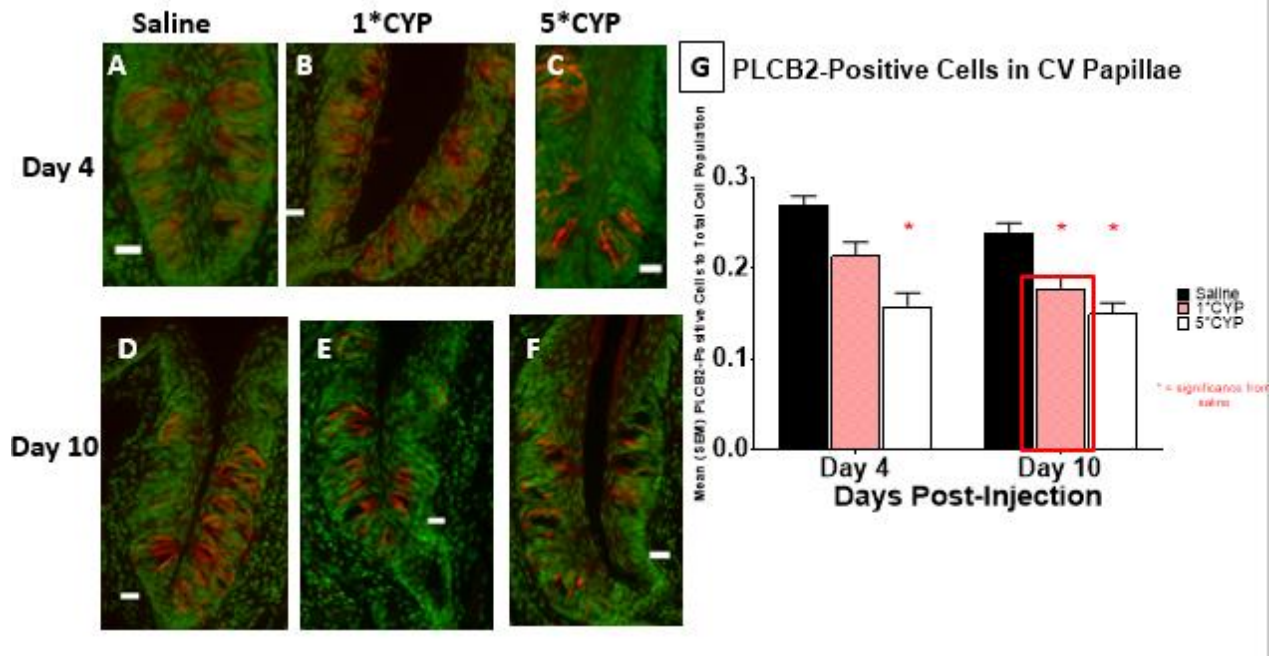


Figure 4: Mean (SEM) of PLCβ2-positive cells to total cell population in circumvallate papillae (CVP) for CYP groups. (A-C) PLCβ2-positive cells (red) in CVP at 4-days post-injection for saline, 1*CYP, and 5*CYP. (D-F) PLCβ2-positive cells (red) in CVP at 10-days post-injection for each of the treatment groups (bar = 20 μm). (G) Mean (SEM) PLCβ2-positive cells to total cell population in CVP for each drug condition at day-4 and day-10 post-injection (p<0.05).

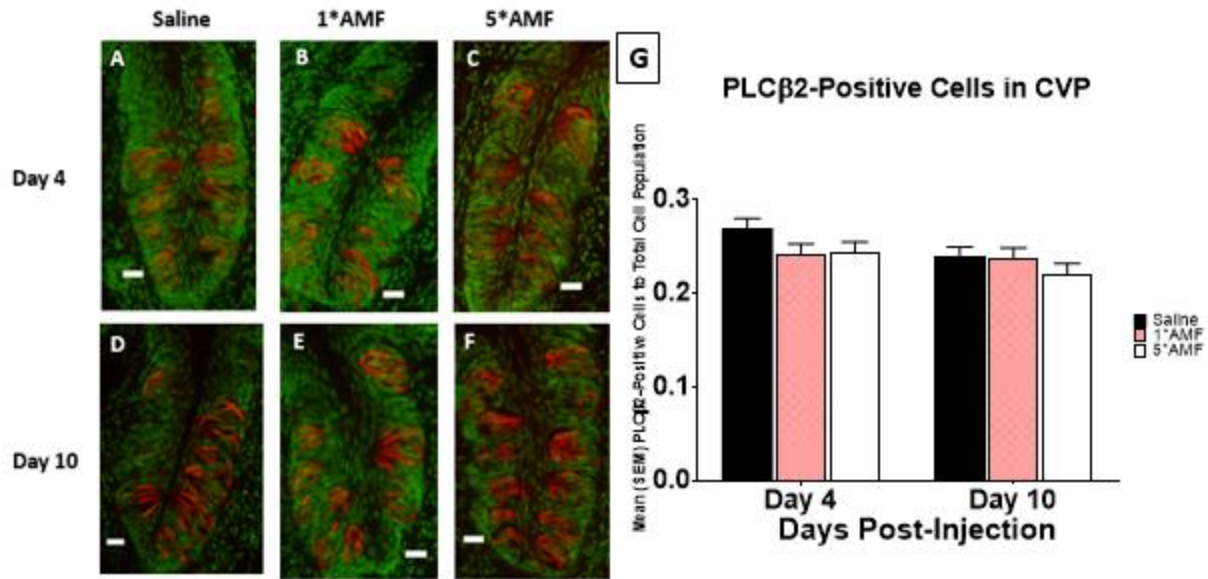


Figure 5: Mean (SEM) of PLCβ2-positive cells to total cell population in CVP for AMF groups. (A-C) PLCβ2-positive cells (red) in CVP at 4-days post-injection for saline, 1*AMF, and 5*AMF. (D-F) PLCβ2-positive cells in CVP at 10-days post-injection for each of the treatment groups (bar = 20 μm). (G) Mean (SEM) PLCβ2-positive cells to total cell population in CVP for each drug condition at day-4 and day-10 post-injection.

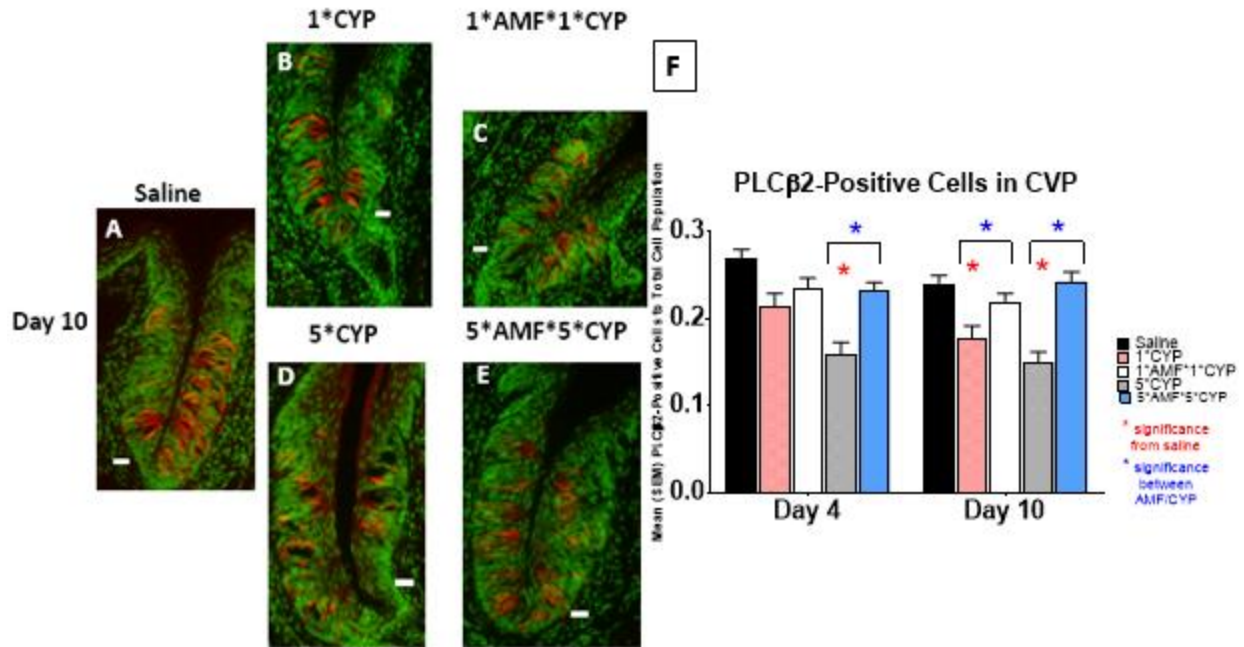
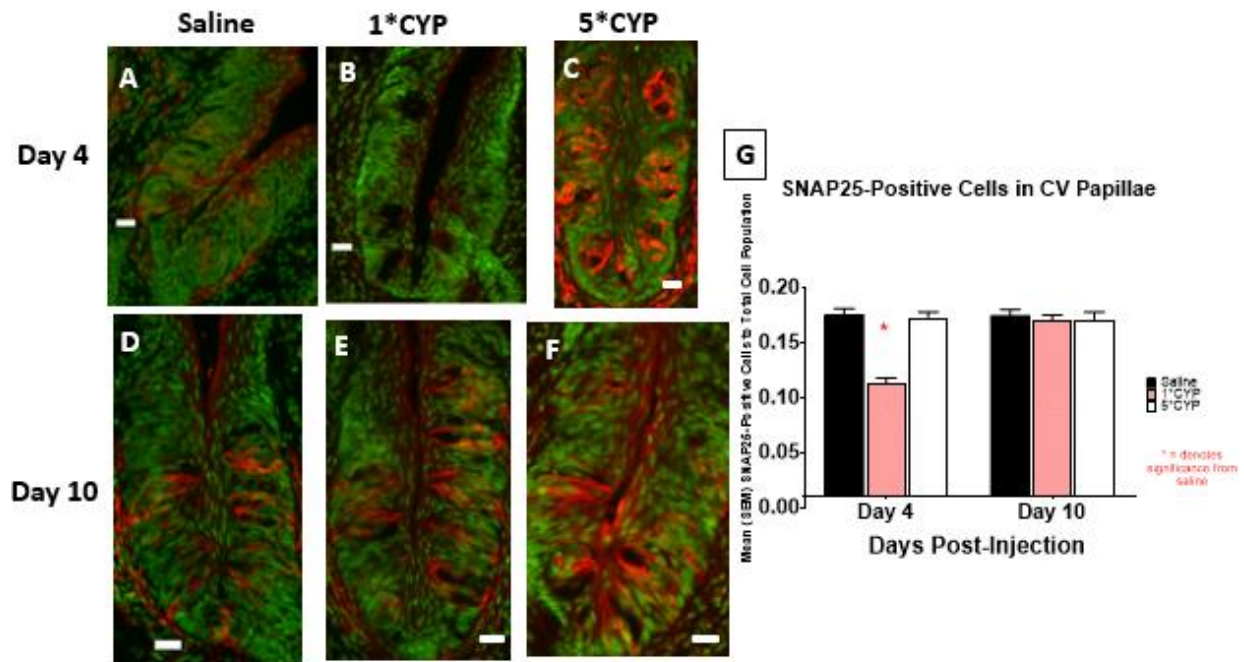


Figure 6: Mean (SEM) PLCβ2-positive cells to total cell population in CVP for AMF*CYP groups. (A-E) PLCβ2-positive cells (red) at 10-days post-injection for saline, 1*CYP, 1AMF*1CYP, 5*CYP, & 5AMF*5CYP (bar = 20 μm). (F) Mean ratio (SEM) of PLCβ2-positive cells to total cell population in CVP for both 4-day and 10-day post-treatment

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*Figure 7: Mean (SEM) of SNAP25-positive cells to total cell population in CVP for CYP groups. (A-C) SNAP25-positive cells (red) in CVP at 4-days post-injection for saline, 1*CYP, and 5*CYP. (D-F) SNAP25-positive cells (red) in CVP at 10-days post-injection for each of the treatment groups (bar = 20 μ m). (G) Mean (SEM) SNAP25-positive cells to total cell population in CVP for each drug condition at day-4 and day-10 post-injection ($p < 0.05$).*

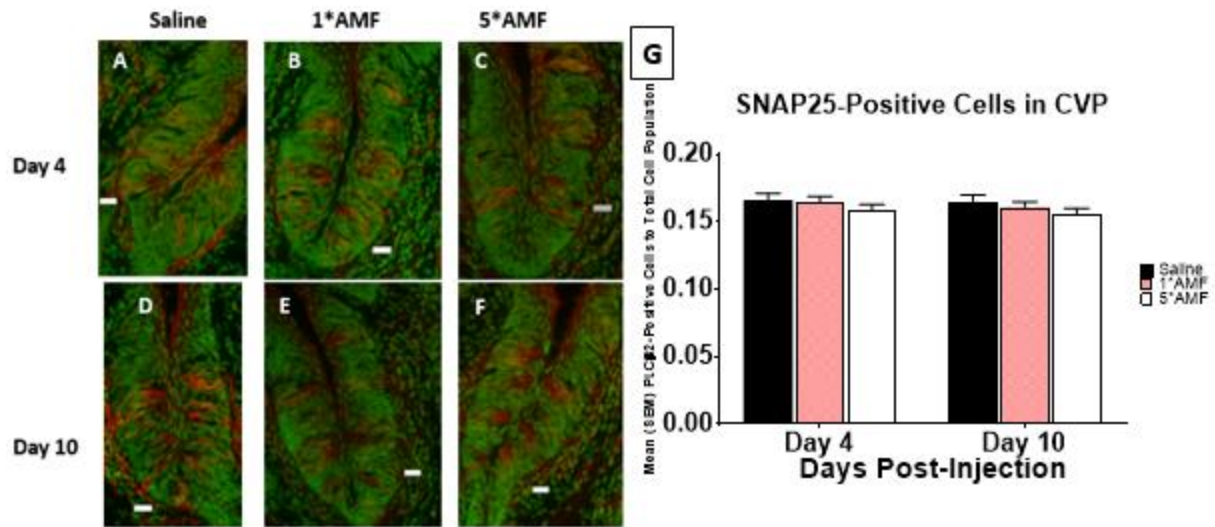


Figure 8: Mean (SEM) of SNAP25-positive cells to total cell population in CVP for AMF groups. (A-C) SNAP25-positive cells (red) in CVP at 4-days post-injection for saline, 1*AMF, and 5*AMF groups. (D-F) SNAP25-positive cells in CVP at 10-days post-injection for each of the treatment groups (bar = 20 μ m). (G) Mean (SEM) of SNAP25-positive cells to total cell population in CVP for each drug condition at day-4 and day-10 post-injection.

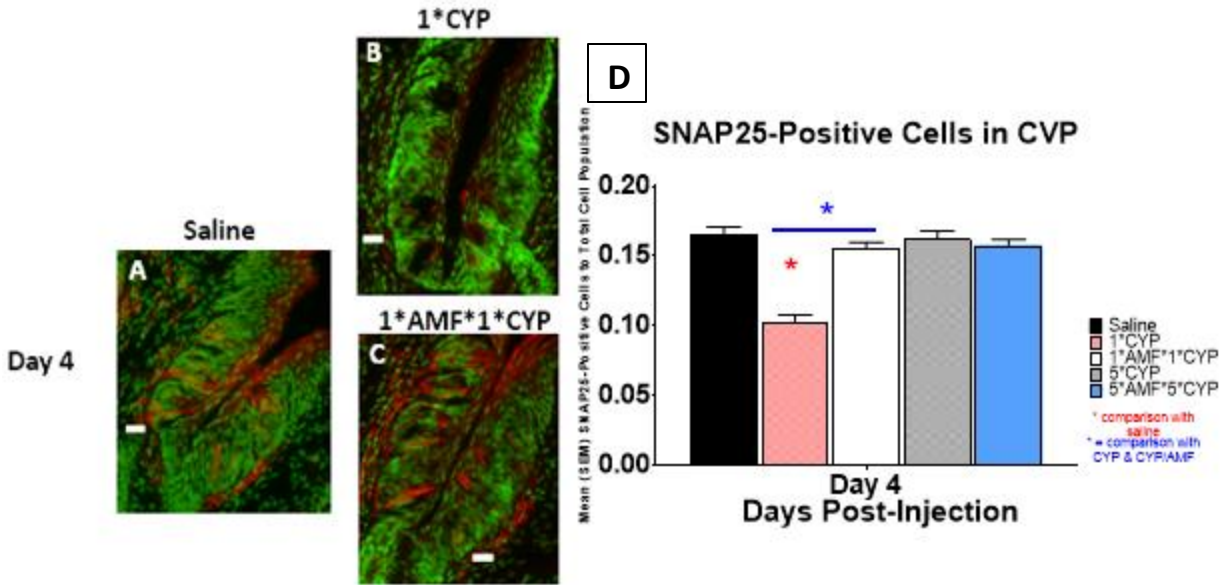


Figure 9: Mean (SEM) SNAP25-positive cells to total cell population in CVP for AMF*CYP groups. (A-C) SNAP25-positive cells (red) at 4-days post-injection for saline, 1*CYP, & 1AMF*1CYP (bar = 20 μ m). (D) Mean ratio (SEM) of SNAP25-positive cells to total cell population in CVP at the 4-day time point post-treatment ($p < 0.05$).