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Understanding the Role of ANO1 in Oral Cancer

Mallary Forrest

UNDERSTANDING THE ROLE OF ANO1 IN ORAL CANCER

By

Mallary Forrest

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University of Florida
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Doctor of Dental Surgery
University of Maryland, Baltimore
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Mallary Forrest

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Fiona Britton, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Dean

Karl Kingsley, Ph.D.
Examination Committee Member

Katherine Howard, Ph.D.
Examination Committee Member

Courtney Coughenour, Ph.D.
Graduate College Faculty Representative

Abstract

UNDERSTANDING THE ROLE OF ANO1 IN ORAL CANCER

By

Mallary Forrest

Dr. Fiona Britton, Examination Committee Chair
Professor of Biomedical Sciences
University of Nevada, Las Vegas
School of Dental Medicine

In 2008, the gene ANO1 was discovered to encode a calcium activated chloride channel. This gene is located on the 11q13 locus, a locus that is commonly amplified in many cancers including cancer of the head and neck. ANO1 is situated in close proximity to genes associated with growth and apoptosis. As rapid proliferation and lack of apoptosis are hallmark characteristics of cancer, growth factors and apoptosis mediators are expected to be altered in cancer. But what does a calcium activated chloride channel have to contribute to cancer's pathogenesis? Is it an active gene in cancer progression or is it simply amplified due to its close proximity to a gene that is driving the upregulation of this locus?

To date, ANO1 has been found to be elevated in many cancers, including cancers of the head and neck. It has been associated with a poor prognosis and a high incidence of metastasis, but its mechanism of action is unclear. Research is relatively new and is limited, especially with its association of head and neck cancer. Perhaps, if ANO1 does have a role to play in cancer

progression, ANO1 may provide a therapeutic target and/or diagnostic and prognostic marker in cancer patients.

The purpose of this study is to attempt to understand more clearly ANO1's role in oral cancer. After ANO1's activity is altered, proliferation and pivotal genes associated with apoptosis will be evaluated in head and neck cancer cells.

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I would like to thank Dr. Fiona Britton for her guidance in this research project. Her continuous support was invaluable and greatly appreciated. Also, a special thank you to my other committee members, Dr. Karl Kingsley, Dr. Katherine Howard, and Dr. Courtney Coughenour.

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Chapter 1: Introduction

Background and Significance

The 11q13 locus is amplified in many different cancers including cancer of the head and neck (Wilkerson and Reis-Filho, 2013). This locus harbors the ANO1 gene (Katoh and Katoh, 2003) which in 2008 was discovered to be a calcium activated chloride channel (Yang et al., 2008; Caputo et al., 2008; Schroeder et al., 2008). Since its discovery there has been a spark in the literature about its presence and association with cancer. To date, ANO1 has been discovered to be amplified in many cancers including head and neck cancer (reviewed in Wang et al., 2017). Its presence has been associated with metastasis and poor prognosis in cancer patients (Ayoub et al., 2010). However, research is relatively new and limited. If ANO1 does play a role in the pathogenesis of cancer, its exact role is uncertain. Perhaps ANO1 could serve as a future drug therapy or as a prognostic marker in cancer patients, but further research is necessary. The aim of this study is to evaluate if ANO1 has a role in proliferation and apoptosis in head and neck cancer. Our approach will be to alter the activity of ANO1 channels with a biochemical inhibitor and evaluate the effect of this inhibition on two characteristic hallmarks of cancer cells, rapid proliferation and loss of apoptotic ability.

Research Questions

1. Is the proliferation of oral squamous carcinoma cells affected by the activity of ANO1 channels?
    - Null hypothesis (H_0): Proliferation of oral squamous carcinoma cells are not affected by the activity of ANO1.

- Alternative hypothesis (H_A): Proliferation of oral squamous carcinoma cells are affected by the activity of ANO1.

2. Is the expression of apoptotic mediators in oral squamous carcinoma cells affected by inhibiting ANO1 channel activity?
    - Null hypothesis (H_0): Inhibiting ANO1 channel activity has no effect on the gene expression of apoptotic mediators in oral squamous cell carcinoma cells.
    - Alternative hypothesis (H_A): Inhibiting ANO1 channel activity has an effect on the gene expression of apoptotic mediators in oral squamous cell carcinoma cells.

Approval

No human subjects or clinical samples were used in this study.

Research Design

Oral squamous cell carcinoma (OSCC) cell lines SCC-4, SCC-9, SCC-15, SCC-25, and CAL27 and a normal keratinocyte cell line, OKF4, will be utilized in this study.

Proliferation Assays

Proliferation assays will be utilized to compare the proliferation of cancer cells following treatment with an inhibitor of ANO1 ion channel activity and function ($T16_{inh}$-A-01). Two treatment concentrations (10 µM and 30 µM) will be tested, and the results will be compared against an untreated control. The data generated will be analyzed using two-tailed t-test for statistical significance.

Quantitative RT-PCR

The oral cancer cell lines will be treated with an inhibitor of ANO1 ion channel function ($T16_{inh}$-A-01; 30 µM) and changes in the gene expression of pivotal apoptotic genes (CASP3 and CASP9) will be compared against untreated cancer cells. Additionally, oral cancer cell lines will be compared to the non-malignant cell line for ANO1 gene expression. RNA isolation will be applied to all of the above cell lines and cDNA will be fabricated. Relative qPCR will be performed using TaqMan™ chemistry and specific primers for ANO1, CASP3, CASP9, and the housekeeping gene GAPDH. Gene expression will be normalized to the housekeeping GAPDH gene within its corresponding cDNA. The fold differences in gene expression will be calculated and data generated will be analyzed using two-tailed t-test for statistical significance.

Independent variable: treatment with ANO1 channel inhibitor.

Dependent variables: cell proliferation, gene expression of CASP3 and CASP9.

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Chapter 2

Inhibition of ANO1 Channels Alters the Cellular Phenotype of Oral Squamous Cell Carcinoma

Mallary Forrest[1], Andrew Bowers[2], Fiona C. Britton[3*]

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[1]Mallary Forrest - Department of Advanced Education in Orthodontics and Dentofacial Orthopedics, University of Nevada, Las Vegas, School of Dental Medicine, 1700 W. Charleston, Las Vegas, Nevada, USA, forrem2@unlv.nevada.edu

[2]Drew Bowers - Department of Clinical Sciences, University of Nevada, Las Vegas, School of Dental Medicine, 1700 W. Charleston, Las Vegas, Nevada, USA, bowera1@unlv.nevada.edu

[3]Department of Biomedical Sciences, University of Nevada, Las Vegas, School of Dental Medicine, 1001 Shadow Lane, Las Vegas, Nevada, USA, fiona.britton@unlv.edu

**corresponding author:*

Fiona C. Britton, PhD, Professor of Biomedical Sciences, University of Nevada, Las Vegas – School of Dental Medicine, 1001 Shadow Lane, Las Vegas, Nevada, 89106.

fiona.britton@unlv.edu. Phone: 702-774-8680. Fax: 702-774-2721

Abstract

Background: Anoctamin-1 (ANO1) is a calcium-gated chloride ion channel. The gene for *ANO1* is located on chromosome 11q13, a genetic locus that is frequently amplified in carcinomas, including head and neck cancer. Reports have indicated that elevated ANO1 may be a biomarker for cancers and a prognostic indicator for poor outcomes. The role of ANO1 channels in oral cancer progression are unknown.

Objectives: The aims of this study were to evaluate if blocking the activity of ANO1 ion channels modulates the proliferation of oral squamous cell carcinoma (OSCCs) and to determine if inhibiting ANO1 function alters the expression of apoptosis mediators in OSCCs.

Methods: Experimental growth assays of OSCCs (SCC-4, SCC-9, SCC-15, SCC-25 and CAL27) were performed to evaluate proliferation over 3 days following treatment with $T16_{inh}$-A-01, a pharmacological blocker of ANO1 ion channel activity. OSCCs were treated with 0 µM, 10 µM or 30 µM $T16_{inh}$-A-01 and vehicle DMSO treatment only. At each time point (1, 2 and 3 days), cells were stained with gentian violet and cell growth was analyzed by absorbance at 630nm. The effects of $T16_{inh}$-A-01 treatment on cellular viability was assessed by the trypan blue dye exclusion assay. Changes in the expression of apoptotic caspase genes, CASP3 and CASP9, were assessed by quantitative RT-PCR following treatment with 30 µM $T16_{inh}$-A-01 for 3 days.

Results: Treatment of OSCCs with $T16_{inh}$-A-01 reduced proliferation significantly in a dose-dependent manner, relative to controls. 30 µM $T16_{inh}$-A-01 produced the greatest anti-proliferative effect that was evident after day 2 in all five OSCC cell lines (57% reduction on average; $p<0.005$). Treatment with 10 µM or 30 µM $T16_{inh}$-A-01 had no effect on OSCC viability. Exposure to 30 µM $T16_{inh}$-A-01 for 3 days induced significant increases in CASP3 and CASP9 expression (3.3 to 6.6-fold) in SCC-4, SCC-9 and SCC-25 cells, while <2-fold RNA increases were observed in SCC-15 and CAL27 cells.

Conclusions: Inhibiting ANO1 channel activity alters the cellular phenotype of OSCCs by significantly slowing proliferation and concomitantly up regulating the expression of apoptotic proteolytic cascade genes. Further studies are required to investigate additional mechanisms through which ANO1 channels may modulate oral cancer progression.

Keywords: ANO1, TMEM16A, $T16_{inh}$-A-01, oral cancer

Abbreviations

head and neck cancer (HNC)

oral squamous cell carcinoma (OSCC)

Anoctamin-1 (ANO1)

cycle threshold (Ct)

quantitative reverse transcription polymerase chain reaction (qRT-PCR)

dimethyl sulfoxide (DMSO)

Introduction

Epidemiologic studies of head and neck cancer (HNC) estimate the five-year survival rate of approximately 66%, claiming the lives of nearly 10,000 people in the United States annually (Siegel et al., 2015). Evidence has demonstrated the most important risk factors include alcohol and tobacco use (Vigneswaran and Williams, 2014), with more recent evidence demonstrating links between oral carcinogenesis and progression with the human papillomavirus (Chaturvedi Rettig and D'Souza, 2015).

One of the genetic hallmarks of carcinogenesis is the amplification of the 11q13 locus, which has been observed in many epithelial-derived carcinomas, including cancers of the head and neck (Ramos-Garcia et al., 2017; Wilkerson and Reis-Filho, 2013). The 11q13 locus harbors several genes including cyclin D1 (*CCND1*), a cyclin protein required for the progression of G_1, fibroblast growth factor (*FGF19*, *FGF4*, *FGF3*), and FAS-associated death domain protein (*FADD*), an adaptor molecule that regulates cell apoptotic signals (Sharma *et al*., 2017). Importantly, the 11q13 region also encompasses the gene encoding Anoctamin-1 (*ANO1*) (Li, Zhang, Hong, 2014; Katoh and Katoh, 2003), the focus of the current study. In the past *ANO1* was also known as *TMEM16A*, *TAOS2*, and *DOG1*.

ANO1 is a membrane localized calcium-gated chloride channel that has been characterized extensively by various independent research teams (Yang *et al*., 2008; Caputo *et al*., 2008; Schroeder *et al*., 2008). Multiple important roles of ANO1 channels in cellular physiology include, the regulation of fluids within secretory epithelia such as saliva (Romanenko et al., 2010; Catalán et al., 2015), regulation of gut mobility (Hwang et al., 2009; Mazzone et al., 2011, Sanders et al., 2012), smooth muscle contraction in arteries of the pulmonary (Leblanc et al., 2015), cerebral (Bulley et al., 2012) and coronary branches (Askew-Page et al., 2019), as well mechanisms of fertilization (Dixon et al., 2012) and modulation of pain perception (Lee et al., 2014).

More recently ANO1 channel function is closely linked to cancer, including oral cancer (Wang et al., 2017; Kunzelmann et al., 2019; Crottès and Jan, 2019), where the amplification and overexpression of ANO1 may be involved in tumorigenesis and cancer progression. The upregulation of ANO1 in HNC cells results in metastatic advantage and is associated with poor prognosis and patient outcomes (Ruiz et al., 2012). Recent genome-wide association studies

identified ANO1 as a novel oncogene and prognostic biomarker in esophageal squamous cell cancer (Yu et al., 2019) and other cancers (Zhang et al., 2021).

During the multistep development of human cancers, including oral tumors, a number of cellular features have been described as hallmarks of cancer (Hanahan and Weinberg, 2011). These hallmarks include, increased proliferation, replicative immortality, the ability to evade cell growth suppressors, resisting apoptotic cell death, inducing angiogenesis, and promoting metastasis (Hanahan and Weinberg, 2011). Data from recent studies has led to the suggestion that ANO1 channels function to regulate several hallmarks of cancer development (Qu et al, 2014, Crottès and Jan, 2019). Investigations of epithelial cancers including HNC, have indicated that ANO1 channel activity is capable of enhancing proliferation and metastasis of cancer cells (Ruiz et al., 2012, Jia et al., 2015). One study reported that overexpression of ANO1 may be sufficient to stimulate attachment, increase spreading and detachment and facilitate invasion, which explain the observation of ANO1 effects on cell migration (Ayoub et al., 2010). Others suggest that ANO1 expression is a key factor in the shift of tumors between either a proliferative state or a metastatic state (Shiwarski et al., 2014). Several studies have demonstrated a correlation of ANO1 expression with apoptosis, such as the finding that ANO1 plays an important role in the process of chemotherapy drugs which induce colorectal apoptosis (Lu et al., 2019) and demonstrating that constructing both ANO1 overexpression and ANO1 silencing cell lines can modulate apoptosis in hepatocellular carcinoma cells (Zhang et al., 2020). Moreover, other studies have also demonstrated that inhibition of ANO1 in prostate and colon cancer cells induces apoptosis (Song et al., 2018; Guan et al., 2016).

The majority of studies investigating the role of ANO1 in cancer have employed molecular approaches, such as small interfering RNA, to reduce ANO1 expression. The contribution of

ANO1 channels to cell physiology has benefited from the identification of blockers of ANO1 ion channel activity. $T16A_{inh}$-A01 was identified as a potent and specific inhibitor of ANO1 in a high through-put drug screening analysis of 110,000 small molecules (Namkung et al., 2011), and evaluated for its ability to inhibit ANO1 chloride currents in human bronchial and salivary gland epithelial cells (Namkung et al., 2011). $T16A_{inh}$-A01 at 10 μM inhibited 60% of the ANO1 chloride current and the proliferation of the cells studied decreased significantly (Mazzone et al., 2012). Moreover, recent studies utilized $T16_{inh}$-A01 to assess proliferation in cancers of epithelial origin such as pancreatic cancer (Mazzone et al., 2012), HNC (Duvvuri et al., (2012), prostrate and colon cancer (Guan et al., 2016).

Together studies demonstrate that ANO1 is consistently elevated in many types of cancers and may be a prognostic biomarker of poor outcomes in cancer patients. An understanding of the fundamental concepts of how ANO1 functions in these tumors is still limited and reports on its role in tumor progression are inconsistent. In this study, $T16A_{inh}$-A01 will be utilized to inhibit ANO1 function in a number of OSCC cell lines to evaluate effects of ANO1 channel blockage on proliferation and the expression of genes associated with apoptosis.

Methods

Cell culture

The human oral squamous cell carcinoma (OSCC) cell lines SCC-4, SCC-9, SCC-15, SCC-25, and CAL27 used in this study were originally obtained from the American Tissue Culture Collection (ATTC). CAL27 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum (BCS) and 1% penicillin-streptomycin (Fisher Scientific). SCC-4, SCC-9, SCC-15 and SCC-25 were cultured in DMEM/F12 Ham's media

supplemented with 10% BCS and 1% penicillin-streptomycin. OSCCs were disaggregated using 0.25% trypsin/EDTA (Fisher Scientific) according to standard procedures in a Biosafety Level 2 cabinet (NuAire). The nonmalignant normal oral cell line, OKF4, included for comparison, was cultured in keratinocyte–serum-free medium (Ker-SFM) supplemented with 0.2 ng/ml epidermal growth factor (Gibco Life Technologies) and sub-cultured using 0.1% trypsin/0.01% EDTA (Fisher Scientific). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Viability

Cell viability was determined by a dye exclusion assay following staining with 0.4% tryptan blue solution (MP Biomedicals). Cell counts and percentage viability were measured using dual chamber counting slides (Bio-Rad Laboratories) and a Bio-Rad TC10 automated cell counter. Cells were visualized on an Olympus CK2 inverted microscope.

$T16_{inh}$-A-01 blocker of ANO1 ion channel activity

The $T16_{inh}$-A-01 inhibitor (CAS Number 552309-42-9; Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg/ml, aliquoted into light sensitive vials and stored at room temperature. $T16_{inh}$-A-01 was added to the cell culture medium to give final concentrations of 10µM and 30 µM. Inhibitor concentrations were selected based on electrophysiological studies evaluating the effectiveness of $T16_{inh}$-A-01inhibition of ANO1 chloride currents (Namkung et al., 2011; Mazzone et al., 2012).

Proliferation Assays

Cells were plated at a density of 1 x 10^4 cells/mL in Costar 96-well tissue-culture treated flat bottom plates (Fisher Labs). Experimental growth assays of OSCCs were performed to evaluate proliferation over 3 days following treatment with T16inh-A-01 inhibitor. OSCCs were treated with 0 µM, 10 µM or 30 µM $T16_{inh}$-A-01 and vehicle DMSO treatment only. Three replicates of each experiment were performed, which included three rows of n=8, for a total n=24. After each time point; day 0, day 1, day 2 or day 3, cells were fixed with 10% buffered formalin, stained with a 1% aqueous solution of Gentian Violet (Fisher Labs) and washed with 1X PBS. Cell growth was analyzed for absorbance at 630nm on a Elx808 BioTek microplate reader.

RNA isolation

Prior to RNA isolation, OSCC cell lines were plated in six-well plates in triplicate at a seeding density of 0.3 x 10^6 cells/mL. Triplicates of each cell line were dosed with 30 µM of $T16_{inh}$-A-01 and incubated for 3 days at 37°C. Total RNA was isolated from both treated and untreated OSCCs using TRIzol reagent (Thermofisher Scientific), according to manufacturer's instructions. RNA precipitates were resuspended in 20 µL RNAase-free water. The concentration and purity of the RNA samples were determined using a Nanodrop™ spectrophotometer (Thermofisher Scientific) to measure the absorbance at 260 nm and 280 nm. The A260/A280 ratio >1.8 was considered pure and free from contaminants. RNA was stored at -80°C until use.

cDNA Synthesis

cDNA was fabricated from RNA samples using the High-Capacity™ cDNA synthesis system (Applied Biosystems). Equal concentrations of RNA (2 µg) from treated and untreated cells were transcribed in each cDNA reaction to ensure consistency of RNA input for subsequent quantitative PCR analysis. Each cDNA reaction consisted of 2 µg RNA, 1X reaction buffer, 1X dNTPs, 1X random primer and 1 µL of reverse transcriptase and RNAase-free water to a total volume of 20uL. Reactions were incubated in a Mini AmpPlus™ thermocycler (Thermofisher Scientific) for the following times and temperatures: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C (enzyme heat inactivation). Each cDNA was diluted 5-fold with 80 µL RNAase-free water and stored at -20°C.

Quantitative PCR

TaqMan™ Gene Expression master mix (Thermofisher Scientific) was used for quantitative PCR (qPCR) analysis of ANO1, CASP3, and CASP9. Each 20µL reaction consisted of 1 µL cDNA, 1X TaqMan™ reaction mix and 1X specific TaqMan™ primer. The primers and the associated FAM or VIC probes employed are listed below. qPCR reactions were assembled on 96 well optical reaction plates (Applied Biosystems) and real time amplification was monitored on an Applied Biosciences QuantStudio 3 thermocycler (Thermofisher Scientific) with the cycling profile of 2 minutes at 50°C, 10 minutes at 95°C, then forty cycles of 95°C for 15 seconds; 68°C for 20 seconds. No template controls for each primer assay were included. Amplification of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was included and used to normalize gene expression across the different cDNA samples. The fold differences

in gene expression were calculated using the $2^{-(\Delta\Delta Ct)}$ calibrator method (Livak and Schmittgen, 2001), where relative expression or fold change is equal to $2^{-((\text{Mean } \Delta Ct \text{ Target}) - (\text{Mean } \Delta Ct \text{ Calibrator}))}$.

Statistics

Data were exported to Microsoft Excel and compiled using simple descriptive statistics. Data were presented as mean ± standard error of the mean (SEM). Comparisons of change in proliferation or gene expression were analyzed using two-tailed t-tests, which are appropriate for parametric data analysis. A *p* value of < 0.05 was considered statistically significant.

Table 1. qPCR primers and associated probes employed in this study

Transcript	TaqMan Assay ID	Reporter Probe
GAPDH	huGAPDH1	VIC
ANO1	Hs00216121_m1	FAM
ANO6	Hs03805835_m1	FAM
CASP3	Hs00234387_m1	FAM
CASP9	Hs00962278_m1	FAM

Results

ANO1 expression in OSCCs

We performed quantitative RT-PCR (qRT-PCR) of ANO1 RNA expression in cultured OSCC cell lines SSC-4, SSC-9, SSC-15, SCC-25 and CAL27. The gene expression of ANO1 in each amplification reaction was normalized to endogenous GAPDH expression within the same cDNA sample (i.e., ΔCt). The relative ANO1 gene expression in each OSCC was compared with ANO1 expression in OKF4 non-malignant cells using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) and expressed as a fold difference. Our data indicated that ANO1 expression in OSCC is greatly increased in the OSCCs compared to OKF4 cells (Figure 1, panel A). ANO1 expression amongst the OSCCs on average increased by 1530-fold compared to OKF4 and ranged between 1283- and 1804-fold (Figure 1A). We also examined the levels of ANO6 expression (a paralog of ANO1) and the apoptotic genes CASP3 and CASP9 in OSCCs and OKF4 cells. Our qRT-PCR analyses found no significant differences in the expression of ANO6, CASP3 or CASP9 genes in OSCCs compared with OKF4 cells, with the fold differences in expression in the range of 0.7- to 1.2- fold for these transcripts (Figure 1, panel B).

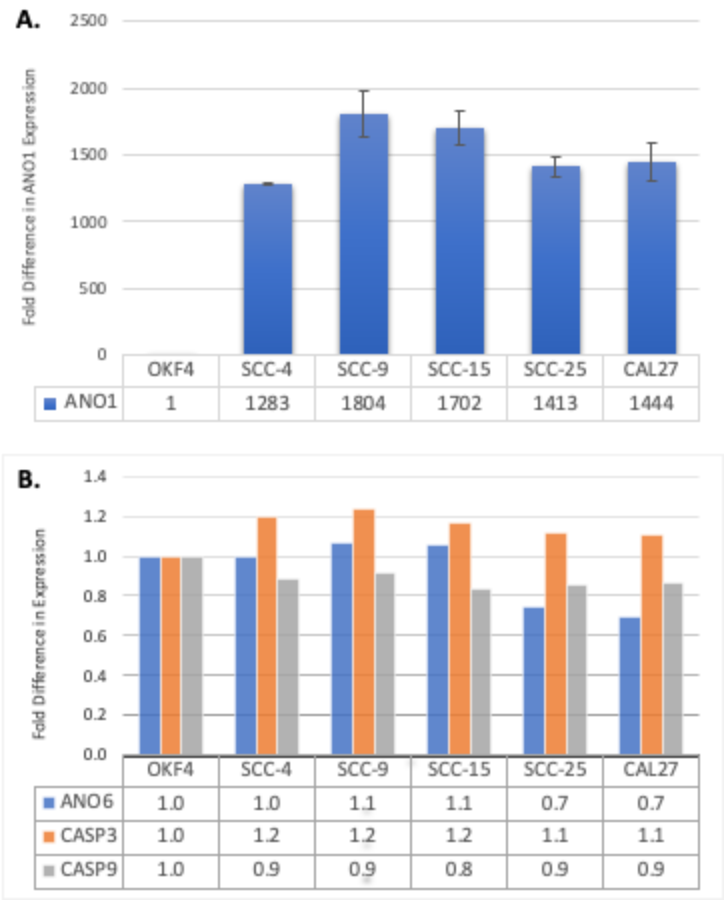

Figure 1: Differences in gene expression in OSCCs compared with non-malignant OKF4. Gene expression was normalized to endogenous GAPDH and expressed as a fold difference when compared with expression in the selected calibrator (OKF4 cells). **A.** Fold differences in ANO1 expression in OSCCs versus OKF4. **B.** Fold Differences in ANO6, CASP3 and CASP9 expression in OSCCs versus OKF4.

Proliferation assays

Assays were performed to evaluate the proliferation of OSCCs following treatment with a pharmacological blocker of ANO1 ion channel activity. Cells were exposed to 10 µM or 30 µM $T16_{inh}$-A-01 and proliferation was assessed over 3 days for comparison with untreated OSCCs and those exposed to DMSO vehicle alone. Figure 2 panels A-E, show the effect of ANO1

channel inhibition on OSCC proliferation. Comparing OSCC proliferation in untreated control (0 μM) with each experimental condition, we found that 30 μM $T16_{inh}$-A-01 significantly reduced proliferation in all OSCCs by day 2 (p<0.005 for SSC-4, SCC-15 and SCC-25 compared with 0 μM; p=0.04 for CAL27, p=0.02 for SCC-9 compared with 0 μM). This anti-proliferative effect remained significant by day 3 (p<0.005 for CAL27, SSC-4, SCC-15 and SCC-25; p=0.01 for SCC-9, compared with 0 μM). The effects of 30 μM $T16_{inh}$-A-01 on proliferation was similar among the OSCCs, although SCC-4 displayed the most sensitivity with a significant reduction in proliferation after 1 day exposure (p=0.005, figure 2B). Exposure to 10 μM $T16_{inh}$-A-01 did decrease proliferation of all OSCCs by day 3, however this reduction was only statistically significant for SCC-15 cells by day 2 (p=0.024, compared with 0 μM) and by day 3 (p=0.016, compared with 0 μM, figure 2D).

Cellular viability

Cellular viability assays were performed on OSCCs that were treated with 10 μM and 30 μM $T16_{inh}$-A-01 for 3 days (Figure 3). Our data demonstrate that the viability of OSCCs cell lines were not significantly altered by the administration of $T16_{inh}$-A-01 ($p = 0.88$, n=3).

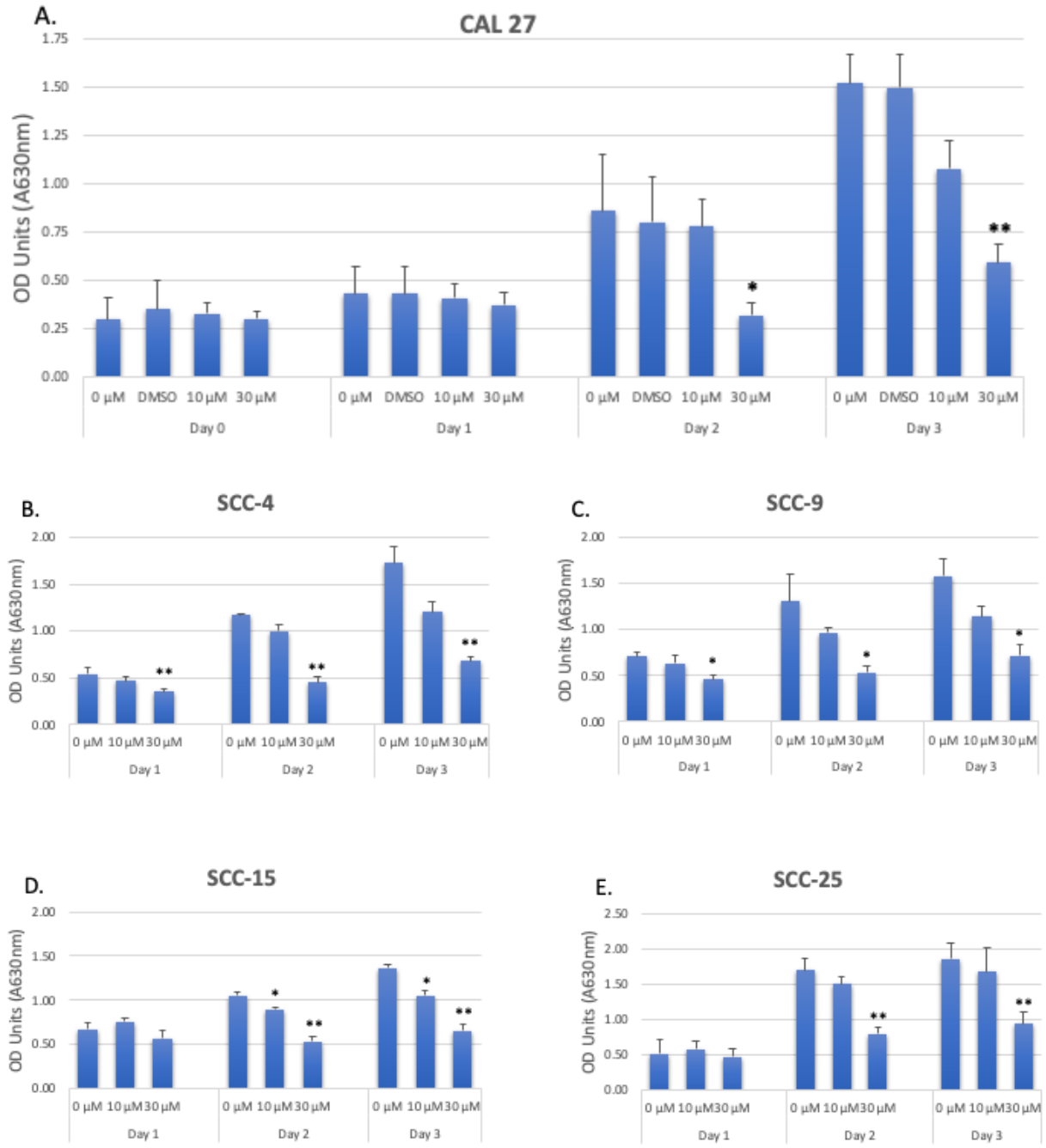

Figure 2: Effects of T16$_{inh}$-A-01 channel blocker on OSCC cell proliferation. **A.** CAL27 proliferation assay showing average A_{630nM} values ± SEM at day 0, 1, 2 and 3 of treatment with 0 µM, 10 µM and 30 µM T16A$_{inh}$-A01 or DMSO only. **B-E.** Proliferation assays for SCC-4 (B), SCC-15 (C), SCC-9 (D) and SCC-25 (E) treated with 0 µM, 10 µM or 30 µM T16A$_{inh}$-A01 over

3 days (Days 1-3). Data shows the average ± SEM from 3 identical experiments. Data generated was analyzed for statistical significance by t-tests of 10 µM and 30 µM treatments vs. 0 µM (*p<0.05; **p<0.005).

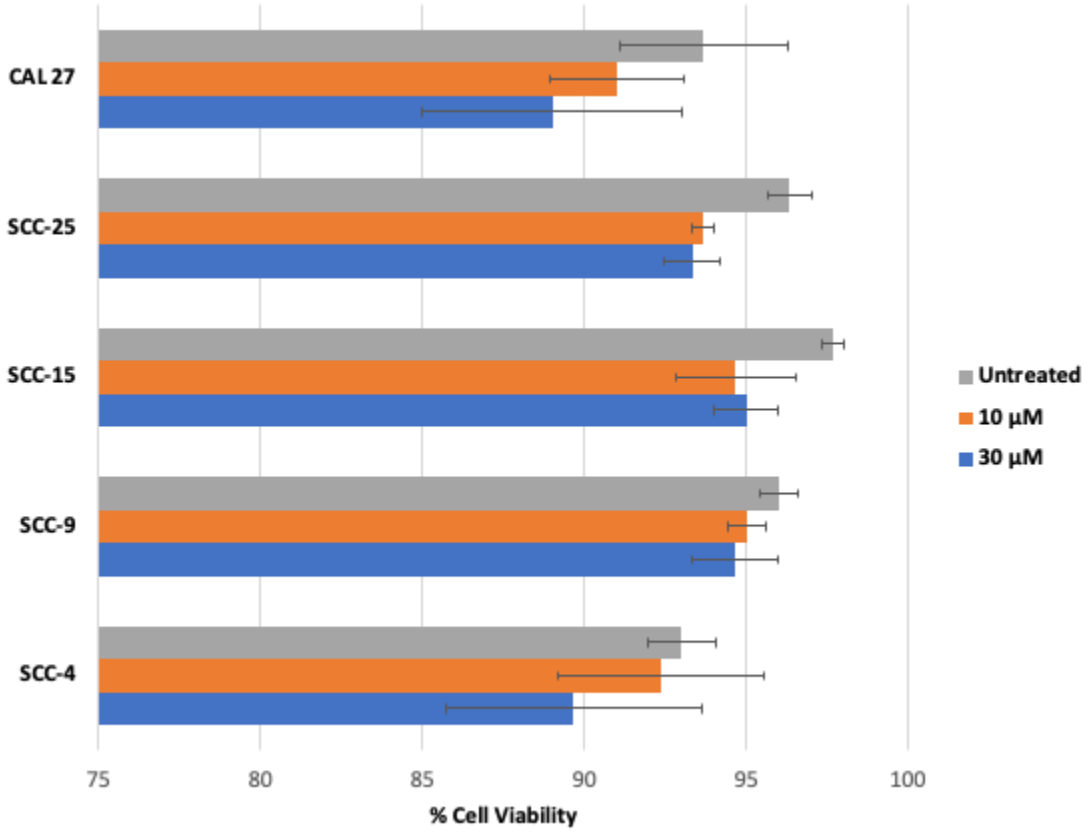

Figure 3: Effects of $T16_{inh}$-A-01 on OSCC viability. Comparison of the viability of SCC-4, SCC-15, SCC-9, SCC-25 and CAL27 cells treated with 10 µM or 30 µM $T16_{inh}$-A-01 for 3 days and compared with untreated (0 µM) OSCCs. No significant changes in cell viability among all OSCCs (p = 0.88, n=3).

qRT-PCR analysis of $T16_{inh}$-A-01 induced expression changes

OSCC cells were exposed to 30 µM $T16_{inh}$-A-01 for 3 days. Following RNA isolation, analysis of ANO1, CASP3, and CASP9 gene expression in inhibitor treated and untreated OSCCs were performed by qRT-PCR. PCR cycle threshold (Ct) values were normalized to GAPDH values within the same cDNA samples and untreated OSCCs were selected as a calibrator for ΔΔCt calculations of fold changes in RNA expression. The expression of ANO1 RNA was unchanged following treatment with $T16_{inh}$-A-01 (Figure 4), as expected since $T16_{inh}$-A-01 inhibits ANO1 channel function on the cell membrane. The fold differences in ANO1 ranged between 0.12- and 0.59- fold among the OSCCs. We did not consider these changes biologically significant, since less than a 2-fold change is representative of only a difference in 1 Ct (Bustin et al., 2009). CASP3 and CASP9 mRNA expression was induced in all OSCCs when ANO1 chloride channel activity was inhibited, but the expression varied amongst the OSCCs. SCC-4 cells exhibited the greatest fold increase in CASP3 expression (6.57 ± 0.07, p=0.02) compared to untreated OSCCs (Figure 4). CASP3 expression increased 3.55 ± 0.17-fold and 3.25 ± 0.1-fold in SCC-9 and SCC-25 cells, respectively (p=0.04). The changes in CASP9 expression varied amongst the OSCCs. The greatest fold increase in CASP9 mRNA was observed in SCC-25 cells (5.87 ± 0.06, p=0.03). SCC-4 and SCC-9 exhibited 3.70 ± 0.06-fold and 3.94 ± fold CASP9 increases, respectively (p=0.03). CASP3 and CASP9 mRNA were not significantly modulated in SCC-15 and CAL27 cells following $T16_{inh}$-A-01 treatment (<2-fold change, Figure 4).

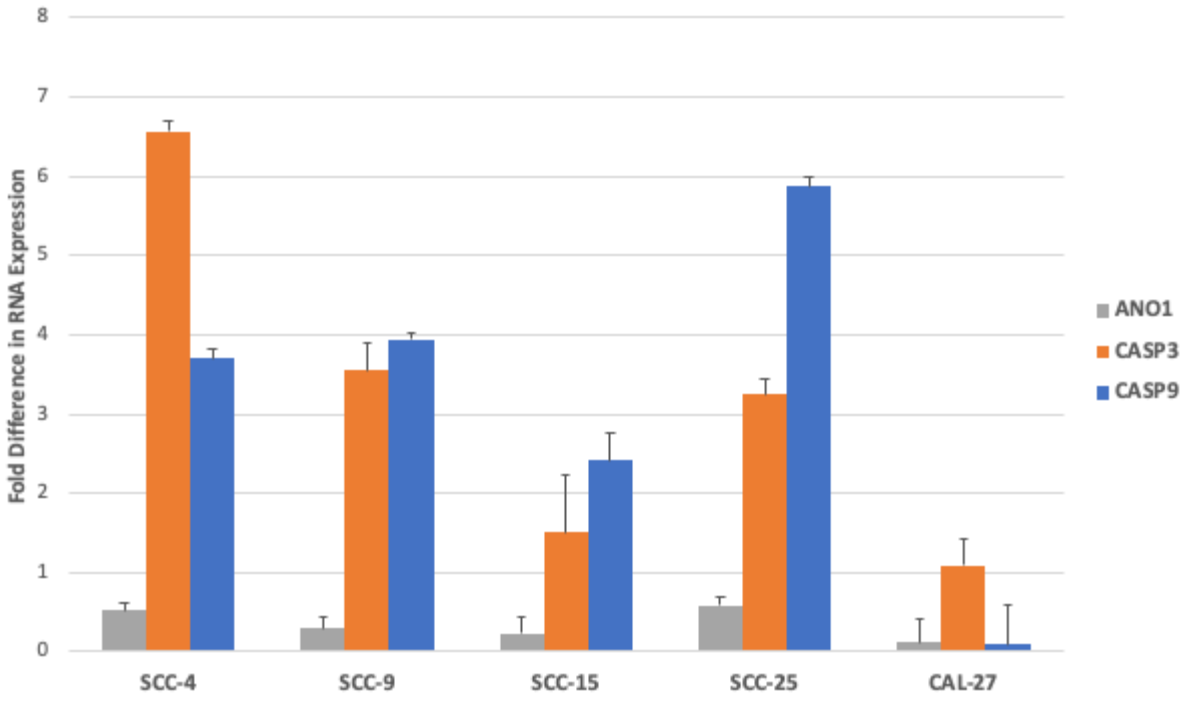

Figure 4: Effects of $T16_{inh}$-A-01 chloride channel inhibition on ANO1, CASP3 and CASP9 gene expression in OSCC. Expression was normalized to endogenous GAPDH and expressed as a fold difference when compared with gene expression in untreated OSCCs (calibrator).

Discussion

This study aimed to evaluate the role that overexpressed ANO1 channels play in the phenotype of oral cancer cells by focusing our examination on changes in OSCC cell proliferation, viability and modulation of apoptosis mediators following inhibition of ANO1 channel activity.

A number of cancers, including head and neck cancer have been reported to overexpress ANO1 due to overamplification of chromosome 11q region, which is the genomic location of the ANO1 gene (Ramos-Garcia et al., 2017). Studies have quantified ANO1 DNA copy number gains and correlated these with increased ANO1 protein in solid tumors using immunohistochemical approaches (Rodrigo et al, 2015). Our qRT-PCR analysis in this study found ANO1 mRNA is greatly overexpressed, on average a 1530-fold increase, in all OSCC cell lines used in this study. This confirms that amplification of the 11q genetic locus and DNA copy number variation in HNC reported by others (Rodrigo et al, 2015), does have a positive correlation with ANO1 mRNA levels in the OSCCs selected for our investigations.

Using a pharmacological inhibitor to block the activity of ANO1 channel membrane proteins, our results demonstrate novel findings regarding the role of ANO1 channels in modulating the cellular phenotype of oral cancer cells. This study suggests that ANO1 channels have a positive effect on the proliferation of oral cancer cells. By inhibiting ANO1 channel function, a significant anti-proliferative effect in OSCCs with the 30 µM $T16_{inh}$-A-01 dosage was observed. This effect was most evident after two days of treatment and the anti-proliferative effect was uniform amongst all five OSCC cell lines included in the study. Inhibiting ANO1 channel function reduced proliferation on average by 57%. This is in agreement of another study in SCC-25 cells (Li et al.,2014), who employed RNA interference to reduce ANO1 expression. The $T16_{inh}$-A01 pharmacological blocker was used to block the activity of ANO1 channel membrane proteins. The majority of investigations of the roles of ANO1 in cancer progression

have utilized gene knockdown approaches with small inhibitor RNA molecules and only a few studies have utilized an ANO1 channel inhibitor. In agreement with our study $T16_{inh}$-A01 induced similar anti-proliferative effects in gastrointestinal stromal tumor cells, pancreatic cancer and prostate cancer (Mazzone et al., 2012; Berglund E et al., 2014, Guan et al., 2016).

Apoptosis is a highly regulated cellular process that is critical for cell growth and tissue development (Elmore, 2007). Loss of apoptosis can result in tumor initiation, growth, and progression and is considered one hallmark of cancer (Hanahan and Weinberg, 2011). Additionally, this study showed that two key mediators of apoptosis, caspase-3 and caspase-9, were concomitantly up-regulated in oral cancer cells by the treatment with the ANO1 channel inhibitor. The levels of upregulation of both CASP-3 and CASP-9 were most significant in SCC-4, SCC-9 and SCC-25 compared to SCC-15 and CAL27, which may reflect differences in signaling pathways by these OSCCs. Perhaps this modulation of apoptotic genes is not the same pathway in all OSCCs, an example of the complex nature of cancer progression (Peltanova et al., 2019) and ANO1 may play different roles in different diseases through different signal transduction pathways. For example, new data now suggest an activation and interaction between the EGFR, TGF-beta, and DNA methylation pathways with ANO1 and apoptosis (Bill et. al, 2015; Dai et. al, 2018; Finegersh et. al, 2015). Zhang et al. (2020) using RNA inhibitors (RNAi/shRNA) to suppress ANO1 expression, suggest that ANO1 may regulate hepatic cancer growth by stimulating apoptosis through the PI3K/AKT-MAPK/p38/ERK signaling pathway. The viability of OSCC treated with $T16_{inh}$-A-01 over 3 days was not significantly altered compared to untreated cells, even though apoptotic caspase genes were upregulated. This observation differs from the study of Zhang et al. (2020). Despite this, our data suggests that

inhibition of ANO1 channel function may induce apoptosis in oral cancer and may limit cancer progression.

Future directions for our studies to evaluate the role of ANO1 in modulating the cellular phenotype of OSCCs would be to include an examination of the effects of ANO1 inhibitor on invasion and migration of OSCCs. Investigations of epithelial cancers, including HNC, have indicated that ANO1 channel activity is capable of enhancing metastasis of cancer cells (Ruiz et al., 2012, Jia et al., 2015). However, it is unclear from the literature if ANO1 is capable of simultaneously augmenting both migration and proliferation in the same cancer cells (Qu et al., 2014; Crottès and Jan, 2019). Some reports suggest ANO1 does not regulate both proliferation and migration in the same cancer cells, such as HNC (Ayoub et al., 2010) and others suggest that ANO1 expression is a key factor in the shift of tumors between either a proliferative state or a metastatic state (Shiwarski et al., 2014).

Conclusion

This study has advanced our understanding of the role ANO1 ion channels play in the cellular phenotype of OSCCs, particularly in terms of the ability of ANO1 to modulate proliferation and alter the expression of apoptotic genes. Further studies are required to investigate the functional consequences of altered apoptotic gene expression and explore additional mechanisms through which ANO1 channels may modulate oral cancer progression.

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Competing Interest

The authors have declared that no bias or conflicts of interest exist.

Author Contributions

MF and AB were involved in data collection and sample preparation. MF and FB were responsible for overall study design and data analysis. All authors participated in manuscript preparation.

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Chapter 3

Summary and Conclusions:

It was recently discovered that ANO1 functions as a CACC and is located on the 11q13 locus, a locus commonly elevated in many cancers including oral cancer. The exact role of ANO1 channels in facilitating oral cancer progression is unknown. The aim of this study was to evaluate if functional ANO1 channels that are overexpressed in OSCCs influence both cellular proliferation and apoptotic gene expression. Using a pharmacological inhibitor to block the activity of ANO1 channel membrane proteins, our results demonstrate novel findings regarding the role of ANO1 channels in modulating the cellular phenotype of oral cancer cells.

First, this study suggests that ANO1 channels have a positive effect on the proliferation of oral cancer cells. By inhibiting ANO1 channel function, a significant anti-proliferative effect of OSCCs with the 30 µM $T16_{inh}$-A-01 dosage was observed. This effect was most evident after two days of treatment and the anti-proliferative effect was uniform amongst all five OSCC cell lines included in the study. Inhibiting ANO1 channel function reduced proliferation on average by 57%.

Second, this study showed that two key mediators of apoptosis, caspase-3 and caspase-9, were concomitantly up-regulated in oral cancer cells by the treatment with the ANO1 channel inhibitor. The levels of upregulation of both CASP-3 and CASP-9 were most significant in SCC-4, SCC-9 and SCC-25 compared to SCC-15 and CAL27, which may reflect differences in signaling pathways by these OSCCs. Our data suggests that inhibition of ANO1 channel function may induce apoptosis in oral cancer and may limit cancer progression. Further studies

are needed to confirm this link and to define the role of mechanism of ANO1 in modulating apoptosis.

In summary, our study highlights the role of ANO1 ion channels in modulating the phenotype of oral cancer cells. This may provide a promising opportunity for therapeutic approaches to regulate ANO1 activity in oral cancer patients.

Based on the findings of this study, both alternative hypotheses can be accepted for research questions 1 and 2.

1. Is proliferation of oral squamous carcinoma cells affected by the activity of ANO1 channel?
    a. Alternative hypothesis (H_A): Proliferation of oral squamous carcinoma cells are affected by the activity of ANO1.

2. Is the expression of apoptotic mediators in oral squamous carcinoma cells affected by inhibiting ANO1 channel activity?
    a. Alternative hypothesis (H_A): Inhibiting ANO1 channel activity influences the gene expression of apoptotic mediators in oral squamous cell carcinoma cells.

Additional Findings:

This study quantified the expression of ANO1 mRNA transcripts in the OSCC cell lines compared to non-malignant cells. This provided important information at two levels.

a) We confirmed that amplification of the 11q13 gene locus, which includes the ANO1 gene, does have a positive correlation with ANO1 mRNA levels in the OSCCs selected

for our investigations. Previous reports of HNC focused on measuring gains in ANO1 DNA copy number and correlations with increased ANO1 protein in solid tumors.

b) We determined ANO1 expression is uniformly high (~1500-fold increase on average) in the OSCCs used in this study and this knowledge eliminated any uncertainty when interpreting our data. Heterogeneity in ANO1 overexpression was reported by other cancer studies such as gastric and made experimental outcomes unclear (Mazzone et. al., 2012).

Our cell viability examination of OSCCs treated with ANO1 inhibitor showed that the percentage viability was not significantly different from untreated cells, so it appears that while ANO1 inhibitor treatment can modulate proliferation in OSCC, it is unlikely that ANO1 inhibitor was toxic to the cells and did not induce necrosis.

Limitations and Recommendations:

Is our inhibitor truly selective for just ANO1?

This project has advanced our understanding of the role ANO1 ion channels play in the cellular phenotype of OSCCs, particularly in terms of the ability of ANO1 to modulate proliferation and alter the expression of apoptotic genes.

One limitation of the project was that we could have expanded our analysis of apoptotic genes to include additional caspase genes involved in the signaling pathways that initiate apoptosis. The

stress pathway/ BCL-2 family pathway activates CASP9 (Elmore, 2007), and our analysis indicated that CASP9 was upregulated in several OSCCs following ANO1 inhibition. The death receptor pathway activates caspase-8 (CASP8) via adaptor proteins that include the Fas-Associated Death Domain protein (FADD), whose gene is also located in the chromosome 11q loci (Akervall et al., 1995). Thus, the inclusion of both CASP-8 and FADD in our qRT-PCR analysis would have been more informative. While the two pathways are largely independent, the activation of CASP3 occurs when the pathways merge (Elmore, 2007), and our analysis did indicate that CASP3 was also upregulated in the same three OSCC cell lines as CASP9 following ANO1 inhibition.

The inclusion of an additional assays, such as the TUNEL assay (Kyrylkova et al., 2012), that would specifically focus on apoptosis, would have been helpful to explore if the induced expression of CASP3 and CASP9 we observed in our study resulted in the characteristics of programmed cell death at the cellular level. Our cell viability examination of OSCCs treated with ANO1 inhibitor showed that the percentage viability was not significantly different from untreated cells, so it is likely that ANO1 inhibitor was not toxic to the cells and did not induce necrosis. However, the inclusion of a TUNEL assay and perhaps longer exposure to $TMEM_{inh}A$-01 (> than 3 days), may have shown cellular indicators of the apoptotic pathway activation. Future directions for our studies to evaluate the role of ANO1 in modulating the cellular phenotype of OSCCs would be to include an examination of the effects of ANO1 inhibitor on invasion and migration of OSCCs. Investigations of epithelial cancers including HNC, have indicated that ANO1 channel activity is capable of enhancing metastasis of cancer cells (Ruiz et al., 2012, Jia et al., 2015). However, it is unclear from the literature if ANO1 is capable of

simultaneously augmenting both migration and proliferation in the same cancer cells (Qu et al., 2014; Crottès and Jan, 2019). Some reports suggest ANO1 does not regulate both proliferation and migration in the same cancer cells, such as HNC (Ayoub et al., 2010) and others suggest that ANO1 expression is a key factor in the shift of tumors between either a proliferative state or a metastatic state (Shiwarski et al., 2014).

Other future directions for this study would be to exam the effects of additional ANO1 inhibitors on proliferation, apoptosis, invasion, and migration in OSCCs. With the increased interest in the cellular functions of ANO1 channels, additional potent and selective ANO1 inhibitors have been designed and synthesized (Ramírez et al., 2020). A series of new 2-aminothiophene-3-carboxamide derivatives, have been tested for ability to suppress proliferation, migration, and invasion of glioblastoma cells (Choi et al., 2020). A new class of ANO1 inhibitor, Ani9, has been tested for anti-proliferative effects in breast and prostate cancer cell lines (Seo et al, 2018), while Kim et al. (2020) developed diaminopyrimidines as novel ANO1 blockers exhibiting anti-cancer effects in lung carcinoma cell lines.

As an alternative to synthetic ANO1 inhibitors, several natural compounds derived from fruits, berries, vegetables, and green teas can have anticancer effects (Giordano and Tommonaro, 2009; Chatelain et al., 2011; Cimino et al., 2012). These natural compounds include major polyphenolic substances, such as epigallocatechin-3-gallate, curcumin, resveratrol and the flavonoids quercetin, tannins, and anthocyanins (Cimino et al., 2012). Indeed, studies by Zhang et al., (2017), have indicate that flavonoids regulate the activity and properties of ANO1 channels. Additional studies examining the modulation of ANO1 channels by dietary

components and their effects on the proliferative phenotype of OSCCs will benefit future therapeutic approaches to regulate ANO1 activity in oral cancer patients.

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Appendix A

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Signature Date

Mallary Forrest Resident

Name (typed) Title

Curriculum Vitae

Graduate College

University of Nevada, Las Vegas

Mallary Forrest

Email: mallaryc10@gmail.com

Degrees:
Bachelor of Science – Integrative Biology, 2006
The University of Florida

Doctor of Dental Surgery, 2011
The University of Maryland, Baltimore

Thesis Title:
Understanding the Role of ANO1 in Oral Cancer

Thesis Examination Committee:
Chairperson, Fiona Britton, Ph.D.
Committee Member, Karl Kingsley, Ph.D. M.P.H.
Committee Member, Katherine Howard, Ph.D.
Graduate Faculty Representative, Courtney Coughenour Ph.D. M.P.H.
Graduate Coordinator, Brian Chrzan, D.D.S., Ph.D.