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Resveratrol Induced Apoptosis in a Human Adenosquamous Carcinoma Cell Line (CAL-27 Cells)

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Philadelphia College of Osteopathic Medicine

Biomedical Sciences

Department of Biochemistry

RESVERATROL INDUCED APOPTOSIS IN A HUMAN ADENOSQUAMOUS

CARCINOMA CELL LINE (CAL-27 CELLS)

A thesis in biomedical sciences by Saquib A. Siddiqi

Submitted in Partial Fulfillment of the requirement for the degree of Master's of Sciences

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Signatory Page for Master's Thesis

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Dedications

First and foremost, I would like to extend by deepest gratitude to my parents, Razi and Shahida Siddiqi. They have been there from the first day, and have always supported and helped attain all my dreams. No matter where I have been, my mother has always cooked food and has always made me feel like I never left home. Her hard work and dedication to her work and family has always been an inspiration. My dad has always been there to listen, rationally, to all the moments of sadness, anger, and happiness. He has reminded me to stay in the middle ground and continuously work hard to achieve my goals. His ability to grow, learn and change is something that I hope to emulate. My goal has always been to make them proud and prove that their hard work has paid off.

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Abstract

Radiotherapy and surgery are the two principal modalities in the treatment of head and neck cancers, and both therapies can result in severe adverse effects and ultimately lower the quality of life. It is of paramount importance to develop reagents that target the cancer cell specifically without affecting the normal non-cancer cells. Using the tongue cancer cell line Cal 27 as a model system, we dissected the molecular mechanism of the resveratrol-induced cancer cell apoptosis. After demonstrating that resveratrol induces the cancer cell apoptosis in a dose- and time-dependent manner, a systemic apoptosis protein array was conducted to identify the resveratrol-induced proteins pertinent to the apoptotic pathways. Ten of the 43 proteins included in the array were up- or down-regulated by resveratrol by about 50 percent. Finally, the activation of caspase-3 and the cleavage of PARP in resveratrol-induced apoptotic cells were confirmed by western blot. We postulate resveratrol induces apoptosis in Cal-27 cells which will render the cells from being able to repair double-stranded-break in the DNA as both P53 and P21 will be up regulated and thus leading to senesce of the cell replication, suggesting that resveratrol could potentially serve as a chemo-preventive reagent.

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Introduction

Cancer is the second most common cause of death in the US, exceeded only by heart disease, and accounting for nearly 1 of every 4 deaths. In the United States this year nearly 2 million people will be diagnosed with cancer and ½ million people, or about 1500 people a day, will die from it. Head and neck cancers make up about 3-5 percent of all cancers (Howlader et al., 2013). Head and neck cancer is the sixth most common neoplasm worldwide with over half million new cases reported each year (Jemal, Siegel Ward, 2008) and head and neck squamous cell carcinoma (HNSCC) is most prevalent. The American Cancer Society approximates that each year there are 28,500 new cases of oral cavity and oropharyngeal cancers in the United States. Of these new cases, an estimated 6,100 will result in mortality. The 5-year survival rate, in all genders and races and at all stages, is only 60 percent. After spreading, the survival rates of regional and metastasized cancers drop to 51.8 and 27.8 percent, respectively (Kademani, 2007).

Oral cavity and oropharyngeal cancers occur most commonly in the tongue (approximately 25% of cases), followed by the tonsils, the lip or the minor salivary glands (each approximately 10% - 15% of cases). The rest are found in the gums, the floor of the mouth, and other sites. Interestingly, 15% of patients who are newly diagnosed with oral and oropharyngeal cancers will also have another cancer in a nearby area such as the larynx, the esophagus, or the lung upon further examination. Of those who are cured of their oral or oropharyngeal cancer, 10% - 40% will develop abovementioned cancers at a later time (Epstein, Gorsky, Cabay, Day, & Gonsalves, 2008).

In general, head and neck cancers encompass a diverse group of uncommon tumors that frequently are aggressive in their biologic behavior. Thus, detection of oral cancers is critically important, because advanced tumors require more aggressive therapy. However, diagnosis and treatment of oral cancers are particularly difficult tasks due to the sheer aggressiveness of these cancers and the fact that despite improvements in surgical and radiation techniques, as well as advances in chemotherapy, the 5-year survival rates for these patients are still only 50%. Half of the patients afflicted with these cancers are expected to die within the first two years of diagnosis (Bankfalvi & Piffko, 2000). Making the situation even more difficult is the fact that a significant increase in the incidence of oral cancer and its mortality has been observed over the last several decades, with a striking upward trend in younger males, especially in some eastern and central European countries (Soleas, Diamandis, & Goldberg, 1997). Thus, oral cancer is expected to be one of the significant public health problems in the foreseeable future (Bankfalvi & Piffko, 2000).

There are many complications that may arise when traditional therapeutic treatments are used and as expected more intensive therapeutic approaches utilized to improve survival among patients also increase complications. Radiotherapy and surgery are the two principal modalities in treatment of HNSCC, but both result in significant side effects which ultimately affect the quality of life of the patients. For example, radiation can induce unavoidable changes in surrounding normal tissues compromising immune function. Mucocutaneous changes as a result of irradiation can lead to hair loss and mucositis. Taste buds are included in the direct beam of radiation used for most oral cancers, making them especially sensitive to irradiation, and as a result most patients will

develop a partial or, more typically, complete loss of taste during treatment. Salivary function is also lost when major salivary glands are exposed to ionizing radiation. This combination of loss of taste and salivary function can have a detrimental effect on a patient's nutrition. Resultant weight loss can lead to weakness, further anorexia, and susceptibility to infection. Lack of saliva and/or changes in the chemical composition of saliva can cause dental caries and tooth decay. The direct irradiation of teeth may also alter organic and inorganic components of the teeth making them more susceptible to decalcification and lead to dental caries. Infections of the mouth by Candida albicans are seen commonly in irradiated patients and are also related to alterations in saliva. One of the more serious complications of irradiation of the head and neck is osteonecrosis. Bone cells and vascularity may be irreversibly injured leading to intolerable pain or fracture and may even necessitate jaw resection. Chemotherapy alone is not an effective treatment for oral cancers, although some regimens can enhance radiation and surgery. The toxic effects of chemotherapy are usually acute and may add to the morbidity of treatment. Therefore, treatment must often strike a balance between the adverse side effects of chemotherapy and the benefits of increasing response and survival (Kademani, 2007). Preventing or at least minimizing these complications is vital to not only successful rehabilitation, but also, quality of life. Efforts to explore innovative mechanism-based targets and approaches for the management of cancer are currently ongoing.

Chemoprevention and treatment via ingestion of natural or synthetic agents with low toxicity that are able to suppress, delay, or reverse carcinogenesis, is being considered as a new dimension in the management of cancer. One of these agents, resveratrol (3, 4', 5-trihydroxystilbene), belongs to a class of polyphenolic compounds

called stilbenes (Soleas et al., 1997). It is a phytoestrogen present in the skin of red grapes and various other food products, with structural similarity to estradiol and diethylstilbestrol (Siemann & Creasey, 1992). Some types of plants produce resveratrol and other stilbenes in response to stress, injury, fungal infection, or ultraviolet radiation (Aggarwal et al., 2004). Plants that produce resveratrol include spruce (picea sp), pine (pinus sylvestris), cranberry (vaccinum sp), mulberry (morus rubra), grapes (vitis vinifera), and peanuts (arachis hypgea). Resveratrol is a fat-soluble compound that occurs in both trans- and cis-configurations (Figure 1). In the recent past, trans-resveratrol has been shown to inhibit cancer initiation, promotion, and progression (Aziz, Nihal, Fu, Jarrard, & Ahmad, 2006). Studies have shown that each gram of fresh red grape skin contains 50 to 100μg of resveratrol, and its concentration in red wine ranges from 10 to 20μmol/L (Jang et al., 1997). Consequently, resveratrol is being considered as an excellent candidate agent for cancer treatment and chemoprevention (Aggarwal et al., 2004).
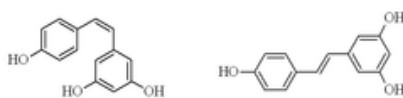

Figure 1- Chemical structures of cis-((Z)-resveratrol, left) and trans-resveratrol ((E)-resveratrol, right)

Scientists became more interested in exploring potential health benefits of resveratrol in 1992 when its presence was first reported in red wine, leading to speculation that resveratrol might help explain the "French Paradox" - the observation

that people from France suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats (Bankfalvi & Piffko, 2000). As a phenolic compound, resveratrol contributes to the antioxidant potential of red wine and may play a role in the prevention of human cardiovascular diseases. Resveratrol has been shown to modulate the metabolism of lipids and to inhibit the oxidation of low density lipoproteins (LDL) and the aggregation of platelets (Bankfalvi & Piffko, 2000). Changes in LDL properties by oxidation of polyunsaturated fatty acids (PUFA) are believed to play a major role in atherosclerosis.

Furthermore, resveratrol obtained from Cussia quinquangulutu (Leguminosae) had a cancer chemopreventive activity in assays representing the three major stages of carcinogenesis (Wilson, Roberts, & Deeley, 1997). It acted as an antioxidant and anti-mutagen and induced the detoxification of carcinogens. It also mediated anti-inflammatory processes mainly by inhibiting cyclooxygenase-1 (COX-1) and hydroperoxidase functions. Resveratrol also inhibited the progression of cancer by inducing cell differentiation. Direct evidence of the chemopreventive activity of resveratrol was assessed by its ability to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture as well as tumorigenesis in a mouse skin cancer model. Likewise, resveratrol inhibited the cellular processes involved in the three stages of tumor development (Wilson et al., 1997). The authors compared the effect of resveratrol in three human breast epithelial cells (immortal estrogen receptor-negative and malignant estrogen receptor-negative or receptor-positive). With each line, the inhibition of proliferation was both dose- and time-dependent. Moreover, treatment with resveratrol reduced the number of viable cells and prevented the exponential growth.

This suggested that resveratrol is a potential chemotherapeutic agent for both hormone-responsive and non-responsive breast cancers. Because cyclooxygenase-2 (COX-2) is important for tumorigenesis, the ability of resveratrol to modulate the gene expression of this enzyme was investigated in human mammary and oral epithelial cells (Wilson et al., 1997). Unlike normal cells, cancer cells proliferate rapidly and are unable to respond to cell death signals that initiate apoptosis. Resveratrol has been found to inhibit proliferation and induce apoptosis in a number of cancer cell lines including prostate cancer (Khan, Adhami, & Mukhtar, 2010), colon cancer (Trincheri, Nicotra, Follo, Castino, & Isidoro, 2007), breast cancer (Sareen, Darjatmoko, Albert, & Polans, 2007), and lung cancer (Whyte, Huang, Torres, & Mehta, 2007). However, the anti-cancer effects of resveratrol on human oral cancer cells have not been well established (Aziz et al., 2006). Furthermore, the protein targets that resveratrol induces to cause apoptosis in oral cancer cells have not been established. Protein targeted therapy is on the forefront of treatment for various diseases like Hodgkin's disease and colon cancer among others, thus establishing the proteins targeted by RSV on oral cancer cells has become more important.

Apoptosis is a recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations. Apoptosis is a vital process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and also in chemical-induced cell death. Apoptotic cell death occurs in two phases: First, a commitment to cell death, and second, an execution phase that is characterized by stereotypic morphological changes in cell

structure, suggesting the presence in different cells of common execution machinery. It affects individual cells and does not induce an inflammatory response. Some morphological changes associated with apoptosis include cell shrinking, cell shape change, condensation of the cytoplasm, nuclear fragmentation, and cell detachment. Some of the functional/biochemical changes include bcl2/bax interaction and DNA denaturing (Kerr, Wyllie, & Currie, 1972). Its morphological features suggest that it is an active, inherently programmed phenomenon, and it has been shown that it can be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological. The structural changes occurring in apoptosis take place in two discrete stages. The first comprises nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane-bound, ultrastructurally well-preserved fragments. In the second stage, these apoptotic bodies are shed from epithelial-lined surfaces or are taken up by other cells, where they undergo a series of changes resembling in vitro autolysis within phagosomes, and are rapidly degraded by lysosomal enzymes derived from the ingesting cells. Apoptosis seems to be involved in cell turnover in many healthy adult tissues and is responsible for focal elimination of cells during normal embryonic development. It is implicated in both physiological involution and atrophy of various tissues and organs. It also occurs spontaneously in untreated malignant neoplasms and participates in at least some types of therapeutically induced tumor regression. It can additionally be triggered by noxious agents, both in the embryo and adult animal (KERR et al., 1972). Inappropriate apoptosis is implicated in many human diseases, including neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, ischemic damage, autoimmune disorders and several forms of cancer (Cohen, 1997).

There are other signs that apoptosis is taking place and can be detected using various techniques for example, cleavage of Poly (ADP-ribose) polymerase by caspase. Not only are cleaved products of PARP detectable, but cleaved proteins of the executioner protein called caspase are also detectable. Poly (ADP-ribose) polymerase (PARP-1) is a nuclear enzyme that catalyzes the transfer of ADP-ribose polymers onto itself and other nuclear proteins in response to DNA strand breaks. PARP assists in the repair of single-strand DNA nicks. It binds sites with single strand breaks through its N-terminal and will recruit DNA ligase III, DNA polymerase beta and a kinase to the nick, in the process of base excision repair (BER). PARP-2 has been shown to oligomerize with PARP-1 and therefore is also implicated in BER. The oligomerization has also been shown to stimulate PARP catalytic activity. PARP-1 is also known for its role in transcription through remodeling of chromatin by PARylating histones and relaxing chromatin structure, thus allowing transcription complex to access genes (Cohen, 1997).

PARP-1, a 116 kDa nuclear enzyme, is cleaved in fragments of 89 and 24 kDa during apoptosis. This cleavage has become a useful hallmark of apoptosis. PARP-1 is also processed during necrosis but a major fragment of 50 kDa is observed instead. According to Gobeil, et al, the cleavage products of PARP will depend on the substance that is causing the apoptosis (Gobeil, Boucher, Nadeau, & Poirier, 2001). In response to DNA damage induced by ionizing radiation, oxidative stress and DNA-binding antitumor drugs, PARPs enzymes add ADP-ribose units to carboxylate groups of aspartic and glutamic residues of target proteins. This poly (ADP ribosylation) activity of PARPs is a post-translational modification that triggers the inactivation of the acceptor protein through the attachment of a complex branched polymer of ADP-ribose units (Cepeda et

al., 2006). In order for apoptosis to take place, the zymogen form of caspase must be activated in order to start cleaving parts of the cell and cause apoptosis.

Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6, which are responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis. There are a number of other mechanisms, aside from activation of the death receptors, through which the caspase cascade can be activated. Granzyme B can be delivered into cells by cytotoxic T lymphocytes and is able to directly activate caspases 3, 7, 8 and 10. The mitochondria are also key regulators of the caspase cascade and apoptosis. Release of cytochrome c from mitochondria can lead to the activation of caspase 9, and then of caspase 3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome C, Apaf-1, pro-caspase 9 and ATP. One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The caspases play an important role in this process by activating DNases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus. The enzyme PARP was one of the first proteins identified as a substrate for caspases. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3. Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic

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cleavage of many key proteins, such as the nuclear enzyme poly (ADPribose) polymerase (PARP), which are cleaved in many different systems during apoptosis (Cohen, 1997).

It has been shown by Attar, *et.al* that resveratrol did cause cell proliferation inhibition as well as DNA synthesis inhibition, of an oral squamous cell cancer line called SCC-25 cells (El Attar & Virji, 1999). However, Attar et.al did not look at apoptosis, or more generally the mechanisms for this inhibition of proliferation of the SCC-25 cells. The SCC-25 cell line is derived from a squamous cell cancer and like other oral cancer cell lines behave very differently from one another. Most tissues within the oral and oropharyngeal cavity have properties of both squamous and gland-like tissues. For example, studies have shown Cal 27 xenographs to be adenosquamous in nature, thus, this cell line would be a better representative for oral cancer than squamous cell cancer cell line. Studying the Cal-27 cells will aid in finding a cell line that is more representative of oral cancers (Jiang et al., 2009).

Cal27 cell line seems to be a better lineage to investigate the effects of resveratrol for inducing apoptosis. These cells were derived in 1983 from the tumor tissue of a 56 year old Caucasian male with poorly differentiated adenosquamous cell carcinoma at the middle of the tongue. Gioanni et al experimented with the Cal 27 cells and found that the doubling time was about 35 hours. These cells were tumorigenic in nude mice. They concluded that the behavior of this cell line in the nude mice and in cultures is similar to that of the original cancers from which it was derived (Gioanni et al., 1988). However, due to variable growth patterns and responses to cytoxicity, the development of an encompassing OSCC model cell line has been challenging. This has presented as a

difficulty to clinicians and has called for the development of more personalized therapies for decades (Gioanni et al., 1988). Therefore, until a strong OSCC model cell line can be established, studying resveratrol effects on OSCC lines for the purpose of clinical treatment will have to be approached sequentially, investigating smaller subsets or cell lineages individually (El Attar & Virji, 1999).

The focus of this study is using the Cal 27 cell line as a model for oral cancer to determine which protein factors are up regulated and down regulated with the treatment of resveratrol. Understanding that quality of life can be drastically diminished using the standard treatments for oral cancer, it is of critical importance to find a means of effective treatment for this cancer that does not reduce the quality of a patient's life dramatically. Removing or damaging parts of the oral cavity due to cancer and or treatment may impact a patient's health, self-image, and cause them to lose their primary means of communication. We know that resveratrol is a natural compound found in many plants and has been used in multiple in vitro and in vivo studies to treat many cancers including, prostate, colorectal, breast, lung, and skin (Anand et al., 2008). If we can show that resveratrol can, not only prevent oral cancer cell proliferation, but by also regulating specific proteins in Cal 27 cells, we can continue to add to the data base of oral cancer cell lines that are susceptible to RSV and eventually find a cell line that will become the ideal model to study oral cancers. Many papers have shown that resveratrol works through the mechanism of apoptosis. As stated earlier we chose Cal 27 cells because Gioanni et al, have described these cells a 'good model for in vitro oral cancer studies' based on doubling time, cell survival, and its response to treatment. Thus, we have chosen to look for conclusive protein markers of apoptosis on Cal 27 cells after they have

been treated with resveratrol. The markers we chose are caspase and poly-ADP ribose polymerase (PARP). In a non-apoptotic cell, PARP protein will be at a baseline level working to repair anything incorrect in the cell. PARP detects and then signals single-strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. PARP activation is an immediate cellular response to metabolic, chemical, or radiation-induced DNA SSB damage. Once PARP detects a SSB, it binds to the DNA, and, after a structural change, begins the synthesis of a poly (ADP-ribose) chain as a signal for the other DNA-repairing enzymes such as DNA ligase III, DNA polymerase beta, and scaffolding proteins such as X-ray cross-complementing gene 1 (XRCC1) (Gobeil, Boucher, Nadeau, & Poirier, 2001). However, it is shown that apoptosis works by cleaving this protein, so it does not interfere with cell death. When a cell is going through apoptosis, we can detect cleaved products of the PARP protein. Bcl-2 protein is the prototype for a family of mammalian genes and the proteins they produce. They govern mitochondria outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak among others) or anti-apoptotic (including Bcl-2 proper, BCL-xl, Bcl-w among an assortment of others) (Cepeda et al., 2006). When a cell is going through apoptosis, an increase or decrease of various markers can be detected.

Depending on the cell line, resveratrol can take an intrinsic or extrinsic pathway of apoptosis (Cohen, 1997). We conducted an assay with the human apoptosis marker kit to show various apoptotic proteins i.e. PARP, BCl-2, and caspase that are either upregulated or downregulated with the presence of resveratrol. We also attempted to look for morphological changes in the cell such as loss of cell membrane integrity by looking for annexin V binding protein, a marker for this process. Finally, we tried to further

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substantiate the apoptosis assay by Western blot analysis. Using the information we gained with the model of tongue carcinoma, we hope to show that resveratrol induces apoptosis while further elucidating its protein pathway of action. Finally, studies using this model for oral cancer may add another piece of information about treating oral cancer with a safe and natural compound.

Materials and Methods

Chemical reagents and cell lines

Resveratrol was purchased from Sigma (St. Louis, MO) and a stock solution (200 mM) was made by dissolving RSV in alcohol and stored at $-20^{o}C$. The CAL 27 cell line was obtained from American Type Culture Collection cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10 percent fetal bovine serum and 1 percent antibiotics at 37°C and 4% CO_2. We used antibiotic-antimycotic (100X) from GIBO (cat 15240). This antibiotic contains 10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 25 μg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone® Antimycotic in 0.85% saline. The cells were cultured in an incubator at 37°C with 4% CO_2 (abcam, 2013).

Addition of 1x 10^6 cells were plated and would equal approximately 70% confluency within 24 hours. We treated the cells with resveratrol at this confluency, as it allowed for adequate time for the cells to fully take up the resveratrol. As the cells divided, the effects of resveratrol could be visualized. Cells were collected at different time points allowing for observation of the effects of resveratrol with varying amounts of time. Cells needed to be sub-cultured after 90 percent confluency was achieved because after this point, cells start dying on their own and, thus we would be unable to differentiate cell death due to resveratrol versus cell death due to stress.

Cell lysate preparation, western blot and antibodies

After treating the cells with resveratrol we incubated them for 2, 6, 24 and 48 hours to determine the amount of time it took for resveratrol to affect the cells. To make

whole cell lysate, cells were washed with cold phosphate-buffered saline (PBS) twice and lysed with EBC Buffer (20 mM Tris-HCl, pH 8.0, 125 mM EDTA and 0.5% NP-40) with proteinase inhibitors. A freeze thaw cycle was done 3 times to induce physical and compositional changes in the cell membranes (Borochov, Walker, Kendall, Pauls, & McKersie, 1987). These compositional changes cause more protein to leak out of the membrane and this can be quantified. Cell debris was removed by high speed centrifugation and lysates were stored at -20°C. Protein concentrations were estimated using the Thermo Scientific Pierce BCA Protein Assay kit. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations over a broad working range (20-2,000 μg/ml). The protein concentrations in cell lysate were inferred from the standard curve (ThermoScientific, 2012) and equal amounts of total proteins (50 μg) loaded into wells in the gel. One lane was reserved for a marker/ladder, a mixture of proteins having defined molecular weights, typically stained so as to form visible, colored bands. Once the samples were loaded, they were separated on a 10% SDS-PAGE gel. We used the "iBlot"dry blotting transfer method. This system blots proteins from polyacrylamide gels in 7 minutes without the need for additional buffers. The "iBlot" device uses disposable blotting stacks with integrated nitrocellulose membranes. The bottom stack includes a 0.2 μm nitrocellulose membrane (Life Technologies Corporation, 2013). Blots were blocked for 2 hours in 5% non-fat milk and incubated with antiserum overnight at 4°C. After washing three times in TBST, the blot was incubated with the secondary antibody conjugated with HRP and detected by Supersignal West Pico Chemiluminescent Kit

(Pierce). The same membranes were stripped and re-probed for either β-actin or GAPDH as internal controls. Western blot provides information about the size of proteins (with comparison to a size marker or ladder in kDa), and protein expression (with comparison to a control such as untreated sample or another cell type or tissue). The antibodies against Caspase-3 and PARP were purchased from Cell Signaling Technology, Inc (Cat#9452, Danvers, MA). This antibody specifically attached to the protein we were looking for.

Initially, the antibody was used in equal parts milk and TBST, which has a small amount of detergent called tween 20, and let it incubate overnight. We eventually changed to using caspin, the agent in milk responsible for the blocking when mixed with PBS. The primary antibody, which is the specific antibody mentioned above, attached to the protein and formed an antibody-protein complex with the protein of interest. After overnight incubation, we took the blot out of the primary antibody, and washed it with TBST for 30 minutes. We put the secondary antibody in caspin or milk. The secondary anti-body was an antibody-enzyme conjugate. The secondary antibody was an antibody against the primary antibody. The conjugated enzyme allowed visualization of the complex. Finally, to see the enzyme, we incubated the blot it in a reaction mix called SuperSignal West Pico Chemiluminescent from Thermo Scientific (lot #KK139345A). If everything worked properly, we saw bands wherever there was a protein-primary antibody-secondary antibody-enzyme complex, or, in other words, wherever the protein was.

Annexin V-FITC assay

The Annexin V-FITC fluorescence Microscopic Kit (51-8074KC) was purchased from BD Biosciences Pharmingen (San Jose, CA). The assay was conducted according to the manufacturer's instruction. Briefly, $1x10^6$ cells were seeded to the 4 well chamber slide system and incubated for 12 hours to adhere the cells to the plate before different concentrations of RSV (0μm, 10μM, 25μM, 50μM and 100μM) were added and incubated for 24 or 48 hours. Cells were wash twice with 1X PBS and stained with Annexin V-FITC (diluted 1:10 in 1X Annexin V Binding Buffer) for 15 min at Room temperature. Cells were washed with 1X Binding Buffer and visualized under light and florescence microscope.

Apoptosis antibody array

The Human Apoptosis Antibody Array Kit (Cat# AAH-APO-1-2) was purchased from the RayBiotech, Inc. (Norcross, GA) and the assay was conducted according to the manufacturer's instruction. In our experiment, two sets of Cal-27 cells, (approximately $1x10^6$ cells) were plated and one plate was treated with 200μM of resveratrol for 24 hours and the other was the control. Briefly, the control or RSV-treated CAL 27 cells were lysed in 1 x Lysis Buffer provided by the kit with addition of protease inhibitors. Lysates containing about 200μg total proteins were incubated with the array membranes over nigh at 4^oC with gentle agitation. After washing twice with 1x Washing Buffer, the membranes were incubated with the Cocktail of Biotin-Conjugated Antibody Mix overnight at 4^oC with gentle agitation, followed by 1.5 hours incubation with the HRP-conjugated Streptavidin at room temperature. Finally, the signals were detected when the

chemiluminescence substrates were added and visualized by exposing to the X-ray films. The intensities of each array dot were quantified by using the Gel-Pro Imager and normalized against the internal control. Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies (RayBio, 2011).

The Human Apoptosis Antibody Array kit allows for a concurrent detection of the relative levels of 43 apoptosis related proteins in cell lysates. By monitoring the changes in protein levels in different experiments, we can study pathway activation. By doing this experiment, we know exactly which apoptosis protein is the highest in our cell and we can focus on that protein to do all our experiment without spending excess time and effort in performing Western Blotting for random apoptosis proteins that may or may not be showing in our cell.

	A	B	B	D	E	Fas	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
2	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
7	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos
8	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos

Chart 1a Antibody array map showing all 43 apoptosis protein markers. This can be used to correlate specific densities matching specific proteins. As can be seen, there are positive and negative controls that are used to normalize the density and thus, negate any variations in exposure.

Results

Effects of RSV on tongue cancer cell CAL 27 proliferation

The general theme of RSV's effects is inhibiting cancer cell growth and enhancing apoptosis (Meeran, Akhtar, Katiyar, 2009). To determine the effects of RSV on oral cancer cells, we first treated the Cal 27 cells with different concentrations of RSV for different periods. The number of cells in the control (0μM RSV) was set as 100% and the numbers of cells treated with different RSV concentrations (0 to 200μM) were expressed as percentage of the control (Graph 1a). It is demonstrated that the effects of RSV on Cal 27 cell growth and/or apoptosis are dose-dependent and this is consistent with previous reports (Wang, Hudson, Remsberg 2010). Since 25μM of RSV was able to inhibit cell proliferation by more than 50%, we conducted a refined inhibition assay (Graph 1b) and shown that RSV inhibited CAL 27 growth in a dose- and time-dependent manner.
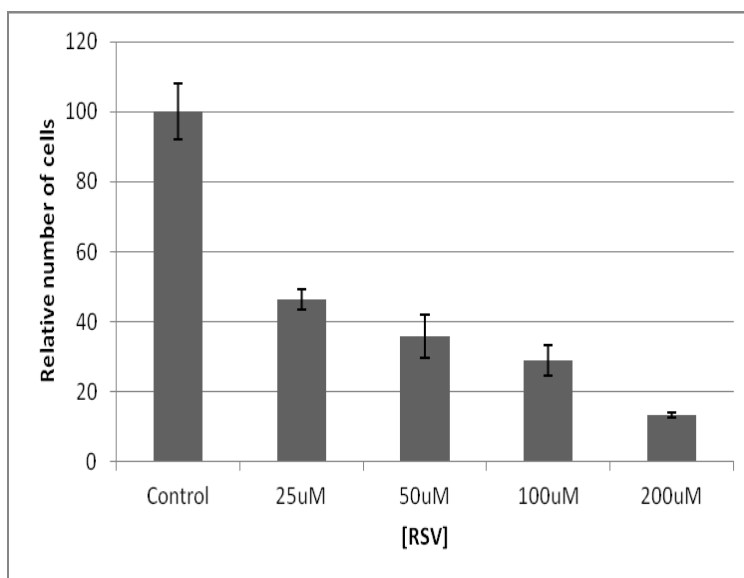

Figure 1a. 5 plates of Cal 27 cells were treated with 25, 50, 100, 200 uM of resveratrol and cells were counted after 24 hours. As the concentration of resveratrol increased, the relative number of cells decreased. This experiment was done in triplicates, and the error bars show variance in the number of cells and the bars show the average of the three plates.
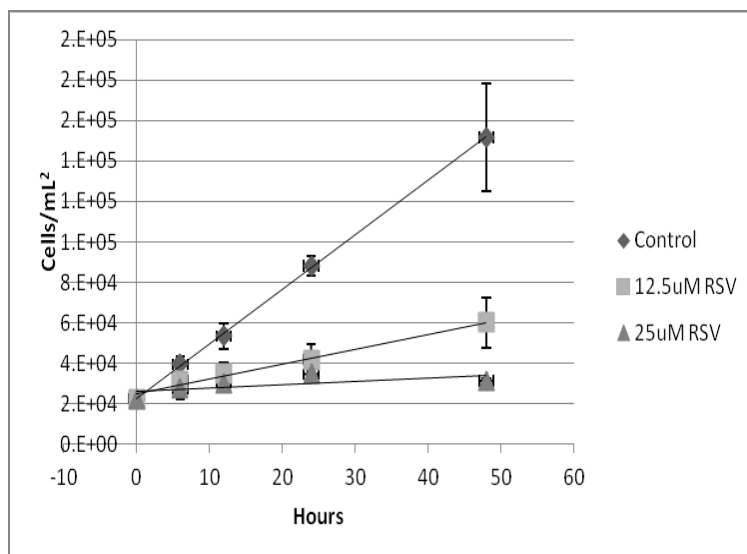
Figure 1b. 2 plates of Cal 27 cells were treated with 12 uM and 25 uM of resveratrol over a period of 60 hours in triplicates. The control group was not treated. This shows both a time and dose dependent effect of resveratrol as well the variance in the number of cells represented by error bars.

RSV induces apoptosis in CAL27 cells

The loss of plasma asymmetry is a key feature of apoptosis. In normal cells, membrane phospholipids are distributed asymmetrically between the inner and outer leaflets of the plasma membrane. For example, phosphatidylserine (PS), an aminophospholipid, is normally present in the inner leaflet (on the cytosolic side) of the plasma membrane. In the early stages of apoptosis changes occur at the cell surface. One of these plasma membrane alterations is the translocation of (PS) from the inner side of the plasma membrane to the outer layer exposing it at the external surface of the cell. Annexin V, a 35-36 kDa, calcium-dependent, phospholipid-binding protein with a high affinity for PS, is used to indirectly monitor PS translocation. Hence, this protein can be used as a sensitive probe for PS exposure upon the cell membrane. We used annexin V labeling in order to test for this loss of cell membrane integrity after we added resveratrol (RSV) to the Cal-27 cells. When PS translocates to the outer surface of the cell

membrane, a fluorchrome attached annexin V protein binds to the PS. Thus, one can use FITC-annexin V and flow cytometry, to identify the population of cells with membrane changes and associated loss of membrane integrity of Cal-27 cells. In normal, healthy cells there is no such translocation of PS and thus, annexin protein cannot bind and cannot be visualized. In this experiment, we were able to visualize time- and dose-dependent effects of RSV on Cal -27 cells. The fluorescence image of the cell membrane shows the annexin V binding to the PS in the subpopulation of the cells going through apoptosis. As seen in the Figure 2a and 2b, the cells that did not receive treatment with RSV did not show any significant fluorescence, suggesting that apoptosis was not occurring.

Results in figure 2a show that cells started undergoing apoptosis at 25uM concentration, bright green indicates that a considerable amount of PS had translocated to the outer surface of the membrane. Looking at the light microscope picture, we can see an intact cell membrane, differentiating it from the process of necrosis, where the cell membrane will have been torn.   The amount of Annexin V-FITC positive cells and the cell morphology can vary according to the cell type, model system, treatment type, or time after apoptosis induction.
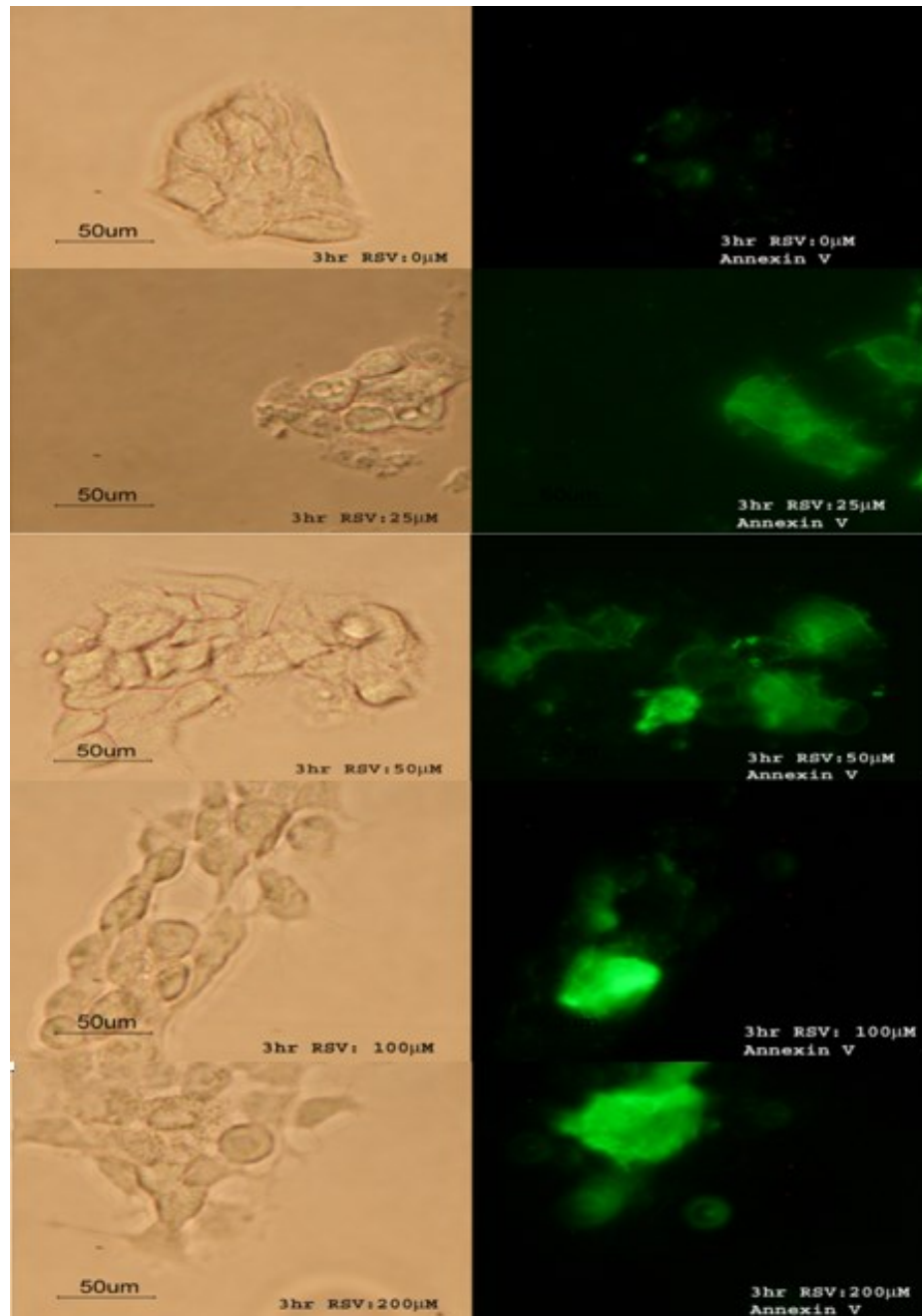
Figure 2a

This panel shows Cal-27 cells collected at 3 hours after treatment with (from the top to bottom): 0, 25, 50, 100, 200uM RSV. On the Left: Cal-27 clusters visualized through a light microscope. On the Right; the same cluster of cells visualized with the flow cytometer. The top panel, which was the control, had no florescence. However, as the concentration of resveratrol increased, the florescence also increased indicating that the cell membrane integrity of many of the cells has been lost. The last panel show a cluster of cells growing very close to each other, with certain cells growing on top of each other.
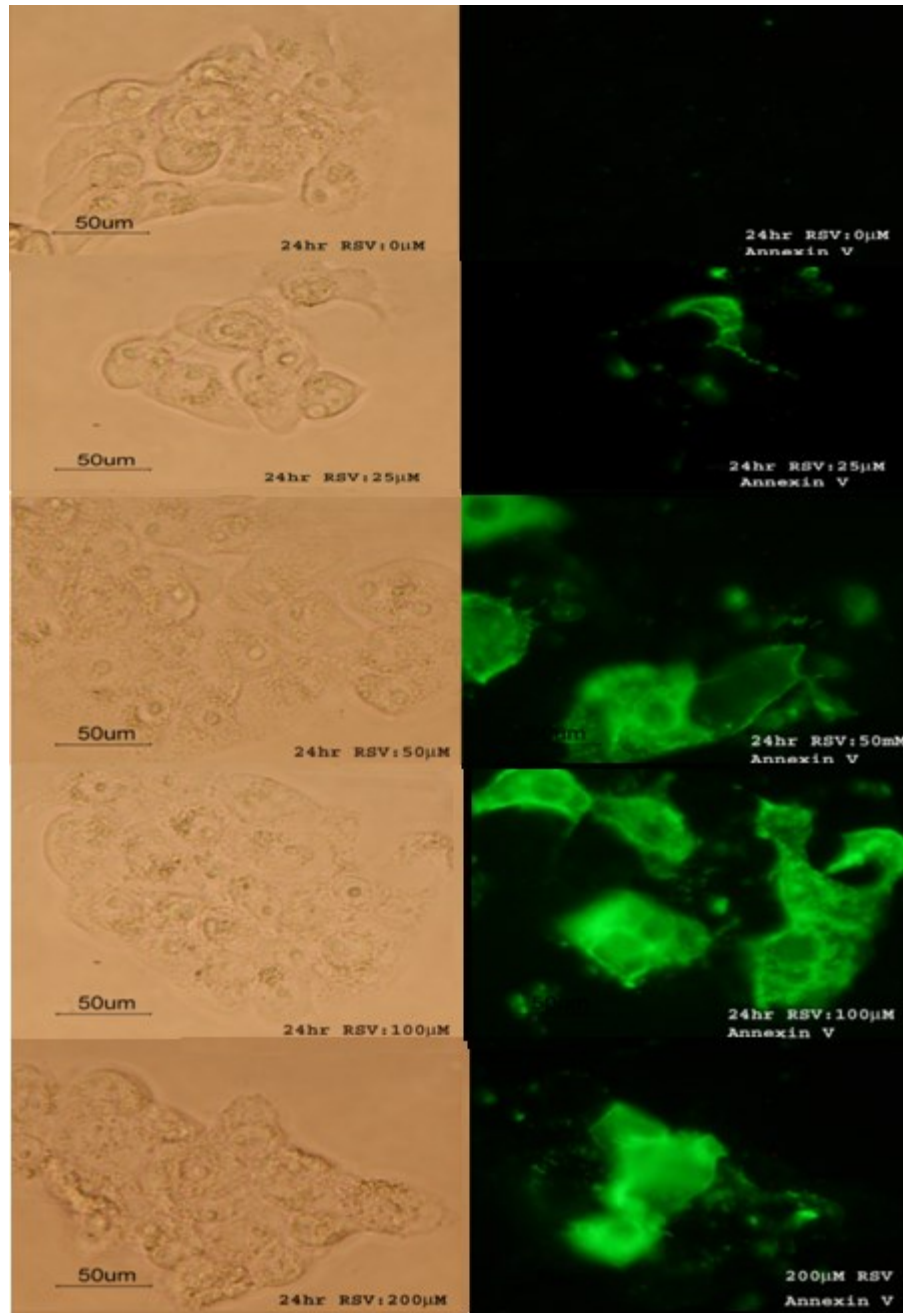
Figure 2b

This panel shows Cal-27 cells collected at 24 hours after treatment with (from the top to bottom): 0, 25, 50, 100, 200uM RSV. On the Left: Cal-27 clusters visualized through a light microscope. On the Right; the same cluster of cells visualized with the flow cytometer. The top panel, which was the control, had no florescence. However, as the concentration of resveratrol increased, the florescence also increased indicating that the cell membrane integrity of many of the cells has been lost. The last panel show a cluster of cells growing very close to each other, with certain cells growing on top of each other.

RSV effects on levels of protein in the apoptotic pathway

As we can see from Figure 3, which was adopted from Murphy, et.al, 2008, there are various pathways for the cell to go through apoptosis. The final step of apoptosis is proteolysis of pro-caspase-3 into activated caspase-3 which will cause irreversible changes to PARP, which subsequently will lead to DNA nicking and cell death. As seen in the figure, there are multiple pathways and enzymes that are either working to keep the cell alive or to cause cell death. As the balance changes either in cell proliferation or cell death, certain proteins and pathways are upregulated and others are downregulated.
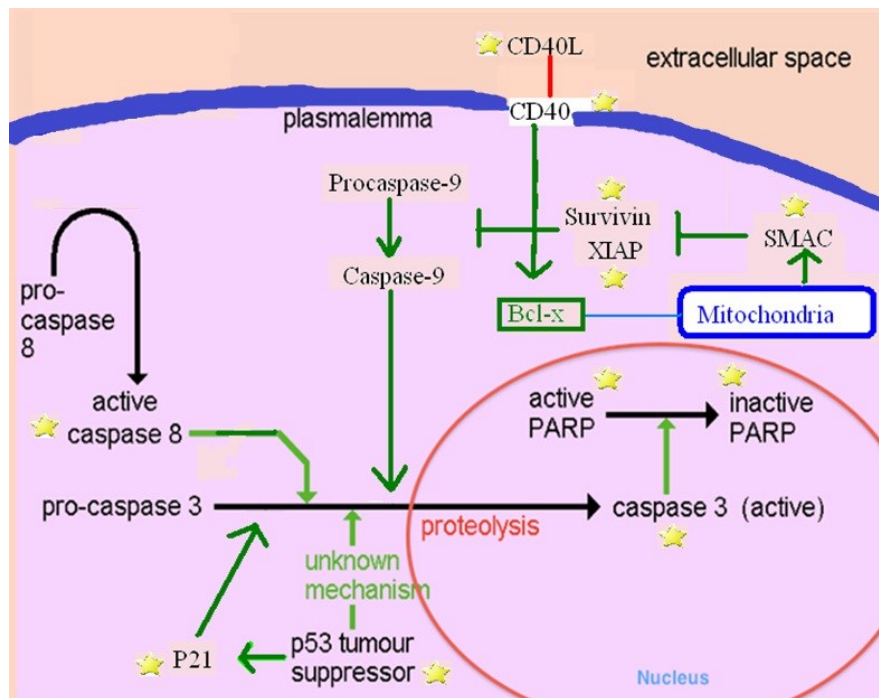

Figure 3. This figure is adopted from Murphy et,al. represents a cell, including the extracellular space, cytoplasm, and nucleus of the cell. Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in DNA repair. Caspase-3 is the executioner caspase and once activated through one of various pathways, it translocates from the cytoplasm into the nucleus and cleaves PARP leaving the cell without one of the most important mechanism to protect and repair itself, leading to apoptosis (Murphy, Perry, & Lawler, 2008).

Before further studying the mechanisms of RSV-induced CAL 27 cell apoptosis, we choose to conduct a systemic search of RSV-induced expression of different protein

factors in the apoptotic pathways. We took advantage of the availability of the Human Apoptosis Antibody Array Kit (Ray Bio) which allows a concurrent detection of the relative levels of 43 well established apoptosis-related proteins in cell lysates. The result is displayed in Figure 4. A computer program (Gel Pro Imager) was used to determine the relative densities of each protein by normalizing to the controls on each membrane.
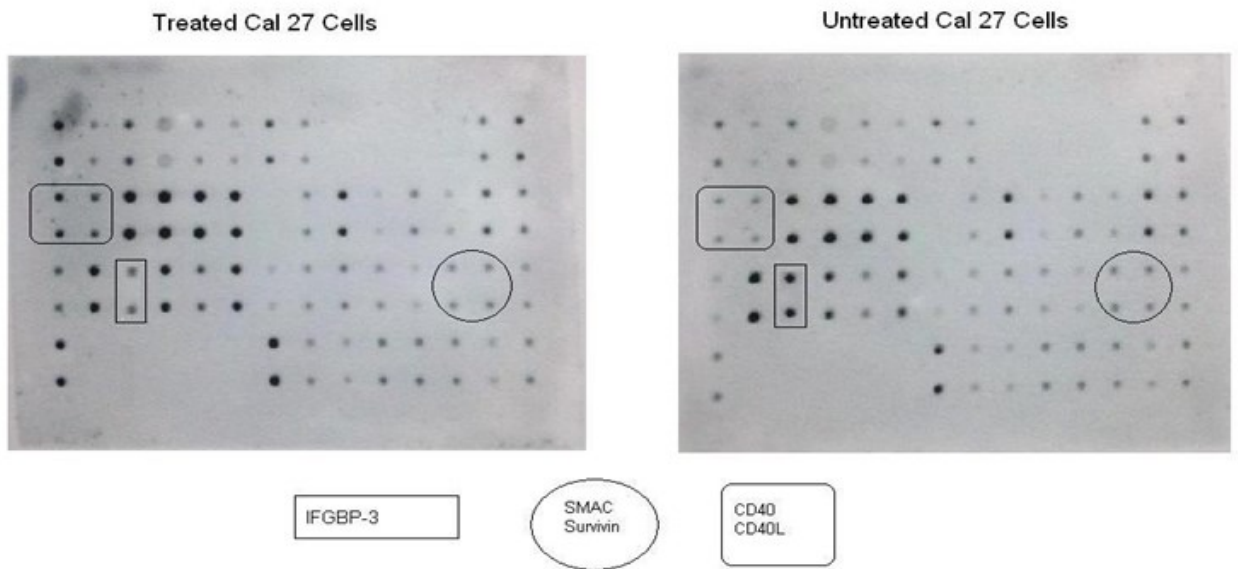

Figure 4. This is the membrane that the proteins have attached to. The highlighted proteins are an example of how each density is related to its protein. The antibodies attach to their respective proteins in duplicates. Using a computer program (Gel Pro-Imager), the relative densities were quantified and normalized to the controls for each membrane.

In order to find the changes of as many proteins as possible, we choose to treat the CAL 27 cells with high concentration of RSV (200μM) for long period (24 hour) of time. Among the 43 protein factors included in the array, 10 (caspase-8, CD-40, CD-40L, HSP-70, IGF-II, p21, p53, SMAC, survivin, and XIAP) of them showed changes (~50 percent change). RSV effect on Cal-27 cells not only upregulated the pro-apoptosis markers such as, SMAC, caspase-8, p21, p53, but it also downregulated the cell survival protiens such as survivin, CD40 and CD40L.

In order to better understand the effects of RSV on CAL 27 cell apoptosis, we categorized the 43 proteins in the array into groups as shown in Chart 1a. As we can see in Graph 2a, Caspase 8 was the most induced factor (more than 3 folds) by RSV. It is well known that Caspase-8 is a member of cysteine-aspartic acid protease family and an initiator caspase that activates executioner caspases (i.e. caspase-3 and caspase-6). On the other hand, both Cd40 and Cd40L were significantly down-regulated by RSV treatment. It has been shown that interaction of CD40 receptors with their ligand CD40L can reduce apoptosis through a caspase-3-dependent mechanism. The interaction between CD40 and CD40L also promote survival by preventing the release of cytochrome-c from the mitochondria and thus prevent the intrinsic apoptotic pathway.
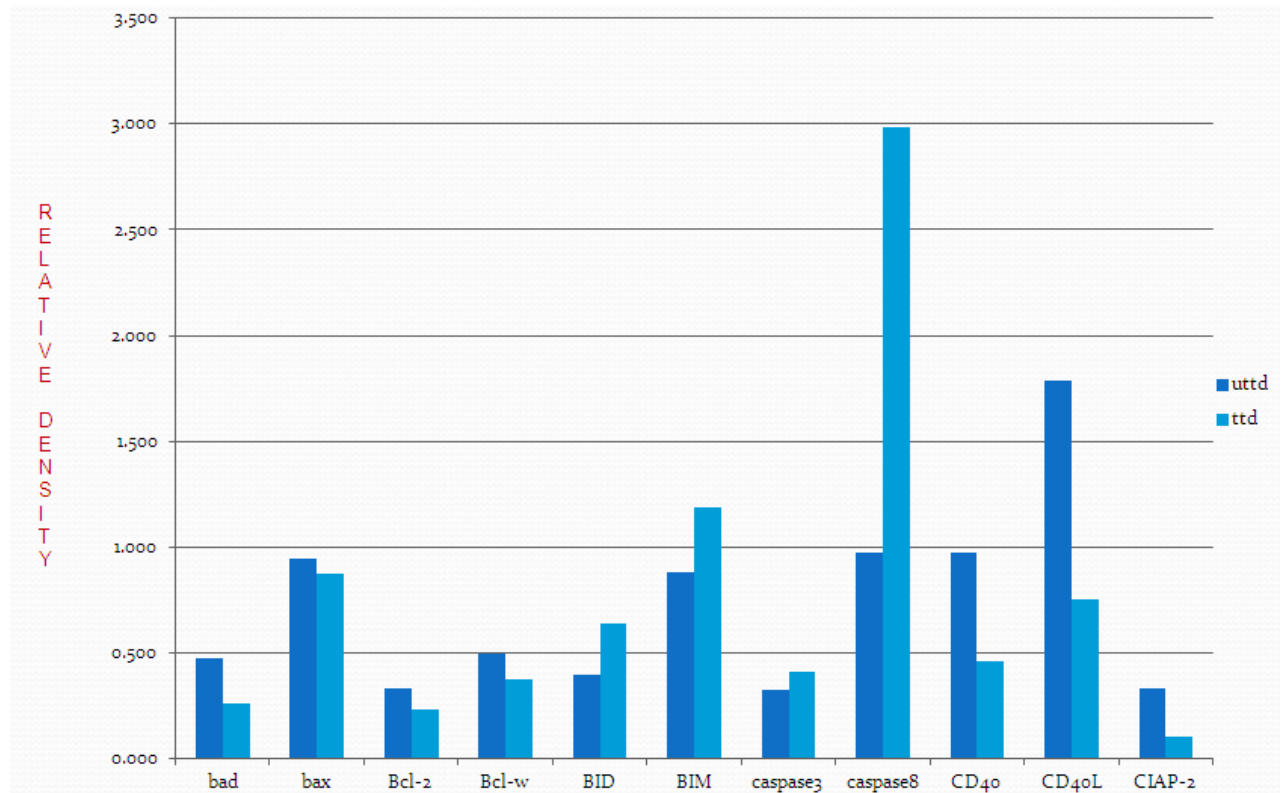
**Graph 2a** In this panel, Caspase 8, Cd40 and Cd40L (~50 % unit) change.

Heat shock proteins are a family of ubiquitously expressed proteins compromising important machinery for protein folding and protection of cells from stresses. Heat shock protein-70 (HSP70) has been shown to be an inhibitor of apoptosis (Calderwood, Xie, Wang, 2010). As seen in Graph 2b, the HSP-70 was down-regulated when the Cal 27 cells were treated with RSV. Insulin-like growth factor 2 (IGF-II) is a protein structurally similar to insulin and it has been suggested that IGF-II enhances apoptosis in osteoblasts (Gronowicz, McCarthy, Zhang, & Zhang, 2004). We found that RSV up-regulates IGF-II in Cal 27 cells.
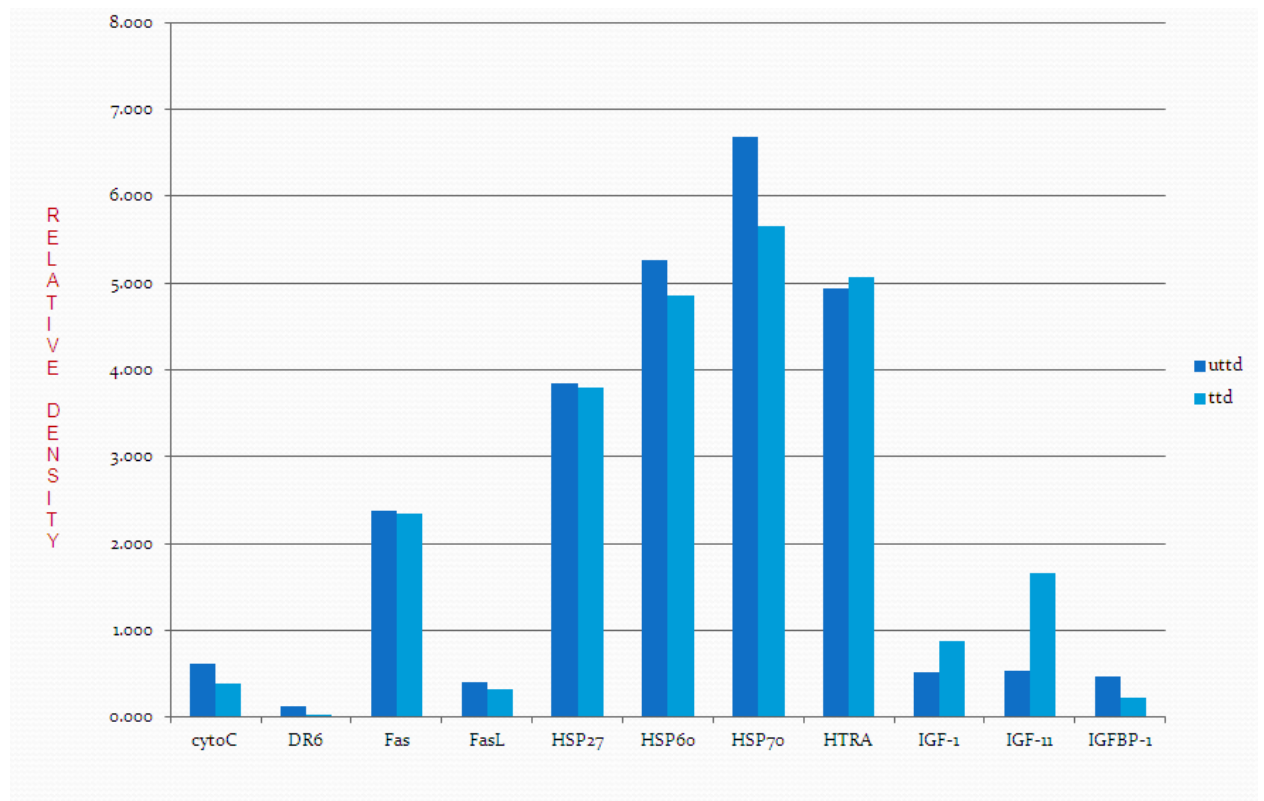
Graph 2b In this panel, changes in HSP70, and IGF-11.

Cyclin-dependent kinase inhibitor 21 (p21) and tumor suppressor P53 are two important factors in promoting cell cycle arrest and senescence (Harper, Adami, Wei, Keyomarsi, & Elledge, 1993). They play vital roles in ensuring that the cell cycle will

not continue unchecked. Both p53 and p21 levels were up-regulated by RSV. In addition, SMAC (small mitochondria-derived activator of caspase) is a mitochondrial protein and leaks to the cytoplasm only when the mitochondrial membrane permeability increased. In the cytoplasm, SMAC binds IAP (inhibitor of apoptosis) and prevent IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed (McNeish et al., 2003). As seen in Graph 2c, when the Cal 27 cells were treated with RSV the levels of p53, p21, and SMAC increased significantly.
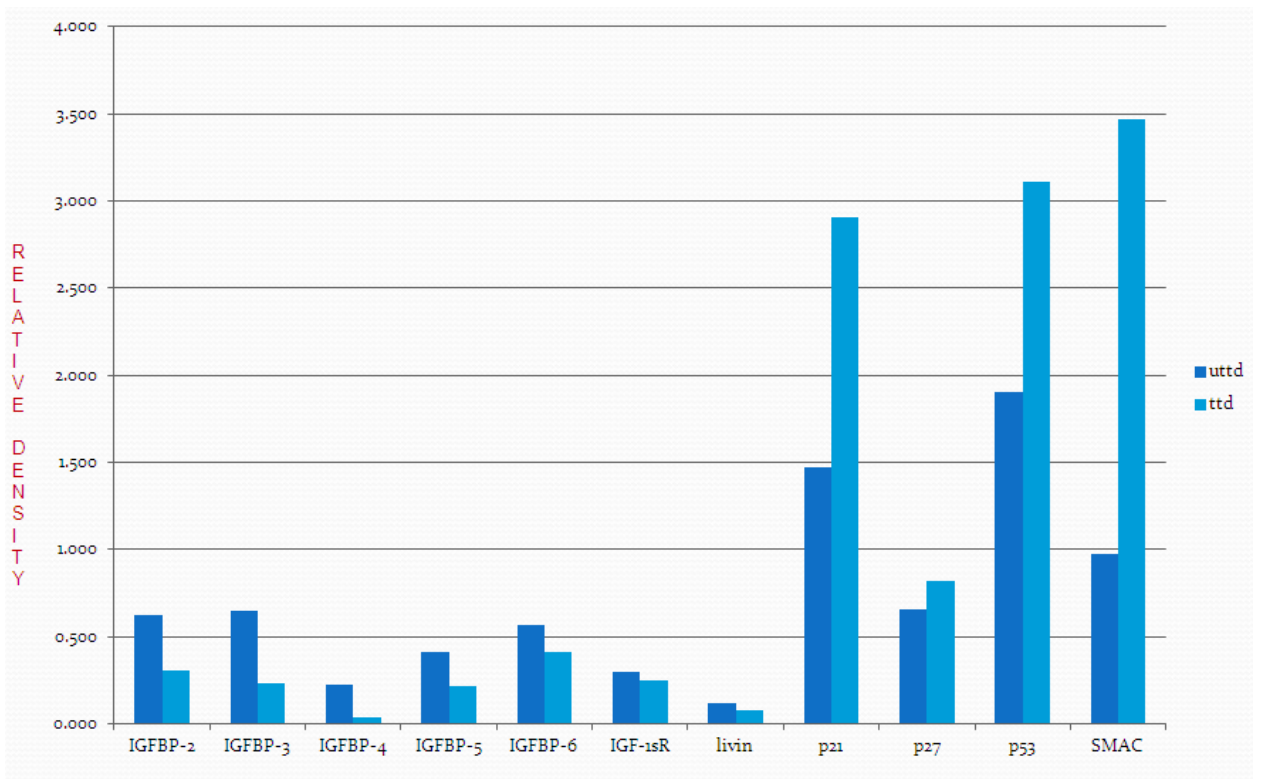
Graph 2c This panel shows changes in p21, p53, and SMAC

Survivin and XIAP are the members of the large IAP family and both of them function to inhibit caspase activation and subsequently inhibit apoptosis (Sah, Khan, Khan, & Bisen, 2006). Survivin is highly expressed in most human tumors and fetal tissues, but is completely absent in terminally differentiated cells (Sah, Khan, Khan, &

Bisen, 2006). As seen in Graph 2d, the level of survivin in the untreated cells is high but down-regulated by RSV. XIAP like survivin is an anti-apoptosis protein, and in our experiment, XIAP, unlike survivin, showed, counter intuitively, an increase after treatment with RSV.
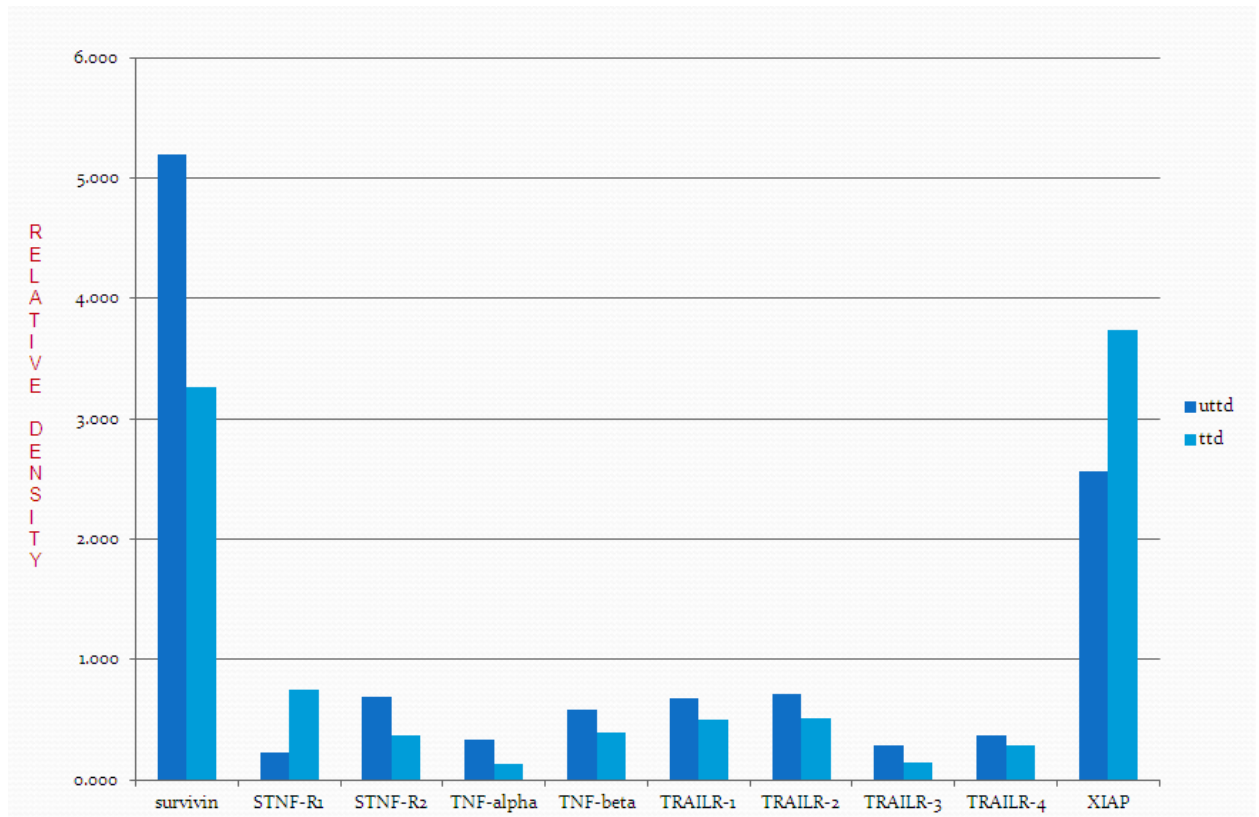
Graph 2d This panel shows changes in survivin and XIAP.

RSV activating caspase-3 and inducing PARP cleavage

Western blot analyses were performed to determine whether caspase 3 activation and PARP cleavage were induced in CAL 27 cells in response to RSV. The cleaved Caspase-3 product was undetectable in the control, but a clear Caspase-3 cleavage product (89 kD) was present in the RSV-treated cells. Activated caspase-3 cleaves DNA

and various proteins, including PARP (Gobeil, Boucher, Nadeau, & Poirier, 2001). Then we conducted western blot using antibody against PARP. The anti-body against PARP was able to detect both the full-length and the cleaved PARP (Figure 6). The full-length PARP protein (116 kD) bands relative density decreased as the concentration of RSV increased. On the other hand, the cleaved PARP product is readily detectable when the cells were treated with RSV at 100µM or higher concentrations.
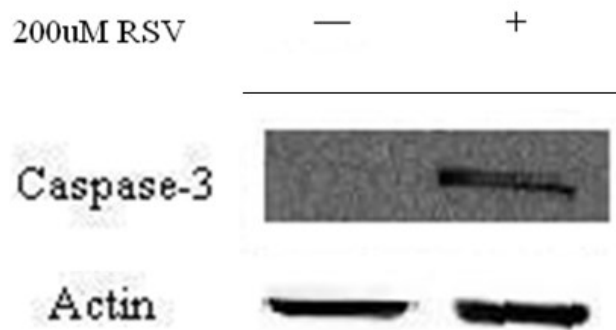

Figure -5 Activated Caspase-3 protein was not present when the cells were not treated with RSV. Addition of 200uM of RSV caused a band. Actin is present as a control.
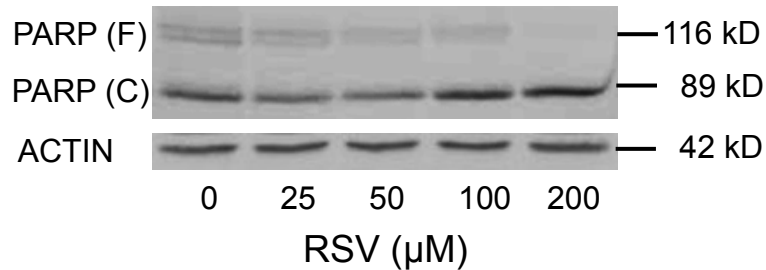
Figure-6. RSV induces cleavage of PARP in Cal27 cells. CAL 27 cells were treated with the indicated doses of RSV for 24 hours. 10ug of whole cell lysate was separated and transferred to a nitrocellulose membrane. PARP Antibody detects endogenous levels of full length PARP (116 kDa), as well as the large fragments (89 kDa) of PARP resulting from caspase cleavage. The antibody does not cross-react with related proteins or other PARP isoforms. (Cell-Signaling Cat#9452)

Discussion

Over the last decade, medicine has made a shift from reactive medicine to proactive medicine. The overall goal of proactive medicine is to assess ways to prevent diseases before they reach the point where the only treatment available is invasive, time consuming, financially burdensome, and potentially dangerous to the patient. RSV has been shown to slow down and stop various cancer cell lines from dividing indefinitely in vitro (Anand et al., 2008). Although other natural products have been proven to be useful in tongue cancer prevention (Tanaka, et al. 2012), there was only one available report about the effects of RSV on HNSCC (Tyagi, Takahata 2011), and this research focused specifically on the relationship between RSV and SMAD4, a transcription factor. In this experiment, Tyagi et al. looked at RSV causing apoptosis in CAL 27 cells through only a specific SMAD4 pathway and did not elucidate a protein pathway of apoptosis. To our knowledge, report presented here is the first systematic analysis of RSV-induced protein expression profile in the tongue cancer cell line CAL 27. Additionally, RSV has not been studied as a treatment of adenosquamous cancer of the oral cavity. The goal of these studies was to determine if RSV would lead to apoptosis and induce cancer cell death in these oral cancer cells and to identify various protein markers in RSV-induced apoptosis.

The Annexin V assay is a widely used assay to show loss of cell membrane integrity. Phosphatidylserine (PS), translocation appears to be a universal apoptotic phenomenon observed in mammalian, insect, and plant cells under the action of most, if not all, triggers of apoptosis (Koopman et al., 1994). It binds preferentially to phospholipid species such as PS, which is normally absent in the outer leaflet of the

plasma membrane and shows minimal binding to phospholipid species such as phosphatidylcholine and sphingomyeline, which are constitutively present in the outer leaflet of plasma membranes. Necrosis, on the other hand, is accompanied by loss of cell membrane integrity and leakage of cellular constituents into the environment. The purpose of this experiment is to show that RSV is inducing the apoptotic process in Cal-27 cancer cells. As shown in the results section, we were able to see fluorescence, suggesting that RSV was causing Cal-27 cell membrane integrity loss and thus, initiation of the apoptotic pathway. The experiment was done in a dose and time dependent manner. We chose 3 hour and 24 hour time periods to observe both early and late changes RSV caused in the cells. We also chose 0, 25, 50, 100 and 200μM of RSV to observe the effect of various concentrations of RSV on the cells. The control group for both 3 hour and 24 hours showed little to no annexin fluorescence, suggesting that the cell membrane of these cells was not changed. As the concentration was increased, both time points showed increasingly higher levels of fluorescence suggesting that a higher level on annexin was being bound to the PS as the apoptosis was initiated by RSV. Due to the fact that we can also see intact cell membranes by light microscope on the left panels on Figure 2a and 2b, it is reasonable to assume that there was no necrosis taking place, as in necrosis the cell membrane immediately loses its integrity and becomes leaky and a the florescence would not be in the circular pattern mimicking the intact cell membrane displayed on the right panels on figure 2a and 2b. Translocation of PS to the external cell surface, however, is not unique to apoptosis and also occurs during necrosis. The difference, though, between these two forms of cell death is that during the initial

stages of apoptosis the cell membrane remains intact, while in necrosis the cell membrane immediately loses its integrity and becomes leaky (BD Pharmingen, 2008).

During apoptosis, there are various protein markers that are up- or down-regulated in order to either help the cell continue with apoptosis or alternatively to promote survival. When a cell is going through apoptosis, various pro-apoptotic markers are up-regulated and anti-apoptotic proteins are down-regulated. Due to the vast amount of markers, we used an apoptosis protein array which detect up to 43 protein markers in one experiment. We used 2 plates of cells, one treated with 200μM of RSV and the other acting as a control. We loaded an equal amount of protein onto the membrane, developed the film and subsequently performed densitometry to compare the relative densities of the proteins and graphed the results. As seen in the results section graph 2a to 2d, there were many apoptosis markers that had a substantial change (defined as 1 unit change). These markers included caspase-8, CD-40, CD-40L, HSP-70, IGF-II, p21, p53, SMAC, survivin, and XIAP. The other markers were not changed significantly due to a number of reasons, one of them being the fact that markers work through different pathways and cascades and at different time points. In our experiment, 10 of the 43 markers showed significant change with 200μM of RSV. Although, we did not specifically focus on pathways, in the future, a good way of explaining these 10 markers would be in a manner that shows the cascade in a chronological order with more certainty than we can infer with our results. For example, we cannot say with certainty that resveratrol caused apoptosis only through an intrinsic or extrinsic pathway or a combination of both. In the future, it would also be a good idea to repeat this experiment multiple times to get statistically significant changes for each apoptosis marker.

Caspase-8 is a cysteine-aspartic acid protease. Caspase-8 exists as a proenzyme and once cleaved is activated into an active caspase, activating on other executioner caspases i.e. caspase 3 and caspase 6. In normal cells, TNF-alpha and SMAC can activate caspase 8 and thus initiate apoptosis (Cohen, 1997). Looking at the graph 2a, we can see that the untreated Cal-27 cells have a baseline level of caspase-8, and once treated with RSV the relative amount of caspase-8 increases nearly three times. RSV, therefore, is likely activating either the intrinsic or extrinsic pathways or a combination of both to increase caspase-8.

Studies done on cancers such as breast cancer and Hodgkin's lymphoma have shown that the interaction of CD40 receptors with their ligand can reduce apoptosis through a caspase-3 dependent mechanism (Voorzanger-Rousselot, Alberti, & Blay, 2006). CD40 and CD40L interaction has been shown to promote survival of the cell by increasing Bcl-X that in turn helps stop the release of cytochrome-c from the mitochondria and thus prevent the intrinsic pathway of apoptosis. In an experiment, Rousselot et.al showed that cancer cells that expressed CD40L are more resistant to cytotoxic agents and cell death. In their experiment, both breast cancer cells and NHL cell lines that were co-cultured with CD40L considerably reduced (from 23-62 percent) apoptosis when cells were treated with anti-cancer drugs (Voorzanger-Rousselot, Alberti, & Blay, 2006). In our experiment, we saw that CD40 and CD40L were downregulated when treated with RSV. This suggests that similar to breast cancer and NHL cells RSV inhibits the anti-apoptotic mechanism in CAL-27 cells.

Heat shock proteins are a family of ubiquitously expressed proteins that are an important part of cell's machinery for protein folding and protection from stress. Heat shock protein-70 (HSP70) has been shown to be an inhibitor of apoptosis. Li et al showed that addition of purified recombinant HSP70 to normal cytosol prevented the cleavage of caspase-3 and DNA fragmentation and thus prevented apoptosis. In Cal-27 cells that were treated with RSV, we saw that HSP-70 was down regulated, again consistent with a decreased resistance to apoptosis (Li, Lee, Ko, Kim, & Seo, 2000).

Insulin-like growth factor 2 (IGF-II), a protein hormone structurally similar to insulin, acts as a growth promoting hormone during gestation (Gronowicz, McCarthy, Zhang, & Zhang, 2004). Gornowic et.al studied the ability of IGF-II to modulate apoptosis in osteoblasts. Over expressed IGF-II produced a dose-dependent increase in apoptosis. In our experiment, when the Cal-27 cells were treated with RSV, we saw an increase in IGF-II correlating with the increase in apoptosis. Cyclin-dependent kinase inhibitor 1 (p21) is a protein that is encoded by CDKN1A gene located on chromosome 6. P21 binds and inhibits CDK thus promoting cell cycle arrest in response to DNA damage or any other stressors that require the cell to go through senescence. If p21 is not working correctly or is inhibited, the cell cycle can continue unchecked, which can subsequently contribute to cancer (Harper, Adami, Wei, Keyomarsi, & Elledge, 1993). In Cal-27 cells treated with RSV, we saw that the p21 level was increased, consistent with RSV inhibition of cell proliferation.

P53 is a tumor suppressor protein that regulates cell the cycle in part by inducing p21. In addition, it can also trigger apoptosis in response to DNA damage by activating

both the intrinsic and extrinsic pathway of apoptosis. P53 controls transcription of proapoptotic members of the Bcl-2 family. It can also activate caspase-9 which helps initiate apoptosis. Its function is vital in ensuring that the cell cycle does not go unchecked and thus, preventing cancer (Fridman & Lowe, 2003). When Cal-27 cells were treated with RSV p53 is upregulated, which again is consistent with RSV inhibition of the cell proliferation and induction of apoptosis.

SMACs (small mitochondria-derived activator of caspase) are mitochondrial proteins and released into the cytosol following an increase in membrane permeability. SMAC binds to inhibitor of apoptosis (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of caspases that degrade the cell (McNeish et al., 2003). When Cal-27 cells were treated with RSV, SMAC protein levels increased, consistent with RSV induced apoptosis through this pathway. Survivin and XIAP are a family of inhibitors of apoptosis (IAP). Survivin, and to a greater degree, XIAP function to inhibit caspase activation which helps negatively regulate apoptosis. The survivin protein is expressed highly in most human tumors and fetal tissue, but is completely absent in terminally differentiated cells. This makes survivin an ideal target for cancer therapy. Survivin expression is also highly regulated in the cell cycle and is only expressed in the G2-M phase. It is known that survivin localizes to the mitotic spindle by interaction with tubulin during mitosis and may play a contributing role in regulating mitosis (Sah, Khan, Khan, & Bisen, 2006). When the Cal-27 cells were treated with RSV, we saw that survivin was downregulated, suggesting that RSV works in a way that reduces the production of the survivin protein which would to help the cell undergo

apoptosis. However, the one discrepancy seen in the proteins that showed a large change in the human apoptosis assay was the increase in XIAP with the treatment of RSV. Like survivin, we would assume that XIAP would also be downregulated if the cells were attempting to undergo apoptosis. One of the explanations for this increase is the fact that XIAP is a very strong IAP and when there is a chemical that will cause apoptosis of the cell, XIAP will begin to increase its production as a last ditch effort to protect the cell from death (Deveraux, Quinnand, 1999).

There are many advantages of using an apoptosis array such as its relatively rapid assessment of apoptotic proteins. It is also relatively economical, and has a high sensitivity; however, there are also some caveats. Although sensitive, it is not very specific. In order to confirm the results obtained from the apoptosis array, we need a more specific test such as a western blot. In order to do this we picked 2 protein markers that have either been historically vital in apoptosis or were upregulated in our apoptosis array. The first marker we chose was caspase-3, which is the active protease, arising from the zymogen form, which translocates into the nucleus and cleaves DNA and other various proteins such as PARP within the nucleus to cause cell death. In western blot analysis (Figure 5) we can see that when the Cal-27 cells were not treated with RSV (in the control group) activated caspase-3 was not detected. However, when the cells were treated with 200μM of RSV, there was an increase in caspase-3 protein which could occur through various different mechanisms (Figure 5). It is reasonable to conclude that through the action of RSV the Cal-27 cancer cells activate caspase-3 to initiate apoptosis and cause cell death.

The other protein we choose was PARP. PARP is an ideal protein to look at because the anti-body we used is able to detect not only the full length PARP but also the large cleaved products. Detecting full length and cleaved products differentiates between cells that have been acted on by RSV and are undergoing apoptosis and cells that have not been acted on by RSV and are not undergoing apoptosis. It can also further substantiate the presence of the activated caspase-3, since caspase-3 is a protease that cleaves PARP. As we can see in Figure 6, as the concentration of RSV increased, the full length PARP protein (116 kD) decreased, suggesting that with higher levels of RSV there was cleavage of PARP. The cleaved PARP products (89 kD) were constitutively present with lower levels of RSV (25μM), but as the RSV concentration increased and more cleavage of PARP occurred, we started to see an increase in protein levels for the cleaved PARP, suggesting that PARP was getting cleaved in response to RSV. The constitutive level of cleaved PARP when the cells were not treated with RSV is one of the confounding variables that don't seem to have a good explanation for. Although, we would expect any cell to have some levels as the cells go through cell death, why there is such a high level is something that needs to be explored in future studies.

Implications and Future studies

Our hope was to increase knowledge of oral cancer, resveratrol and more generally, natural product treatment option for cancer. As stated previously, the current information pertaining to oral cancer and resveratrol is lacking. Numerous studies in oral cancer show that various oral cancer cell lines work in different ways and the closest model to the oral squamous cancer cell (OSCC) line is the Cal-27 cell line. Our goal, which we were successful in achieving, was to show that resveratrol causes apoptosis of Cal-27 cells. Having this knowledge sheds light not only on this specific tongue cancer cell line, but also, more generally on any cancer cell lines that work in a similar fashion. We acknowledge that in the pharmaceutical world, 28,000 new cases of oral cancer is not exactly the most profitable venture to invest in, but the addition of 28,000 new cases on top of the other cancer that RSV already works on would be substantial. The knowledge that RSV causes cell death in this cancer cell line with an idea of the specific protein markers targeted with RSV, will further help establish a stronger and more specific treatment regimen for each individual patient.

From the experiments conducted in this research we found that RSV is indeed causing apoptosis of Cal-27 cells. Future studies should seek to further elucidate the pathway RSV uses to accomplish. If the specific pathway RSV uses can be illustrated, we can further understand the mechanism of oral cancer and how exactly RSV interferes with this pathway, making the treatment of oral cancer more specific, more targeted and faster.

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