# The Effect of Sulindac on Senescence in Retinal Pigmented Epithelial Cells

By

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A Thesis Submitted to the Faculty of

The Wilkes Honors College

in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Arts in Liberal Arts and Sciences

with Concentrations in Biology with a Pre-Med Track

Wilkes Honors College of

Florida Atlantic University

Jupiter, Florida

May 2022

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Chitra Chandrasekhar, and has been approved by the members of his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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Title: The Effect of Sulindac on Senescence in Retinal Pigmented Epithelial Cells

Institution: Harriet L. Wilkes Honors College of Florida Atlantic University

Thesis Advisors: Dr. Chitra Chandrasekhar and Shailaja Allani

Degree: Bachelor of Arts in Liberal Arts and Sciences

Concentration: Biology on a Pre Med-Track

Year: 2022

Age-related macular degeneration is the leading cause of blindness in industrialized countries. Cellular senescence was shown to be involved int he pathogenesis of AMD. We have shown that sulindac, a non-steroidal anti-inflammatory drug, protects normal cells such as retinal cells against oxidative damage through preconditioning mechanism. To test this, we established an in vitro model for senescence in RPE cells by a treatment of hydrogen peroxide (oxidative stressor) for 12 hours followed by incubation for four days. We assayed for senescence by Beta-galactosidase assay to stain senescent cells. Effect of sulindac was tested by pre-treating cells with sulindac at different concentrations for 24 h prior to Tetra Butyl Hydrogen Peroxide treatment. The data shows that Sulindac reduced number of senescent cells in the samples treated compared to the control groups. These findings indicate oxidative damage induces cellular senescence, and sulindac lowers the amount of senescence by protecting from oxidative damage.

## TABLE OF CONTENTS

List of Figures

## Introduction

Oxidative damage was shown to be one of the major factors that play a role in the progression of several age-related diseases such as heart disease, Alzheimer's disease, cancer, and macular degeneration (*1*). Reactive oxygen species (ROS) are highly reactive oxygen free radicals that are generated during the normal metabolic process of respiration, in the mitochondria. Although ROS is essential for cell signaling, higher levels of ROS attack macromolecules in the cell, such as DNA, RNA, lipids and proteins, that leads to oxidative damage, irreversible cell damage and increased cell death(*2*). Cell senescence is the state of permanent cellular division arrest and concerns only mitotic cells(*3*). Cellular senescence is now considered as a hallmark for aging and, studies have shown that ROS promotes senescence(*4*). Oxidative damage drives the cells to become senescent; as we age, the mechanisms to replace senescent cells become inefficient and contribute accumulation of senescent cells that promotes aging process(*5*).

The retinal pigmented epithelial (RPE) layer is one of the major ocular tissues affected by oxidative stress and is known to play an important role in the etiology of age-related macular degeneration (AMD). AMD is an eye disease affecting vision in people over the age of 50 years. Dry AMD is characterized by loss of retinal pigmented epithelial (RPE) cells and, oxidative stress, triggered by increased reactive oxygen species, contributes significantly to the pathogenesis of AMD (1). Oxidative stress induces several cellular alterations including senescence. We initially reported that the drug sulindac can protect both the heart and retinal pigmented epithelial (RPE) cells against oxidative damage by initiating a protective response, similar to ischemic preconditioning (3, 4). In our previous studies, sulindac was tested for protection against oxidative stress-induced damage in an established RPE cell line (ARPE-19)

(*6*). The mechanism of protection appeared to be ischemic preconditioning (IPC) that is triggered by reactive oxygen species (ROS) and involves signaling components such as protein kinase G (PKG), protein kinase C (PKC) epsilon, mitochondrial ATP-sensitive K (+) channel, late-phase IPC markers, iNOS and Hsp70,in the RPE cells that confer protection against oxidative stress (*6, 7*). Activation of the peroxisome proliferator-activated receptor alpha (PPAR-α) is another pathway that was shown to contribute to the protective properties of sulindac(*6*). In that study, low-passage human fetal RPE and polarized primary fetal RPE cells, that mimics the in-vivo condition, were also used to validate the basic observation that sulindac can protect retinal cells against oxidative stress. These findings indicate a mechanism for preventing oxidative stress in RPE cells and suggest that sulindac could be used therapeutically for slowing the progression of AMD (*6*).

RPE senescence may contribute to or/and precede irreversible pathological events in the retina specific for AMD (Age-related Macular Degeneration), such as RPE loss and inflammation. Senescent RPE cells may be excessively damaged, dysfunctional, and capable of overexpression of SASP (Senescence-associated secretory phenotype) which could contribute to the disease pathology (*3*).

AMD & Oxidative Damage: AMD is the major cause of blindness in elderly with an estimated 196 million people globally suffering from AMD in 2020 (7). Loss of RPE cells during the progression of AMD results in degeneration of photoreceptors and loss of central vision (8). Age related oxidative damage results from an altered balance between the increased production of reactive oxygen species (ROS), produced as a byproduct of mitochondrial respiration, and retinal cells are highly susceptible to oxidative damage (1).

Cellular Senescence: Studies have shown that when cells are subjected to insults such as oxidative stress, it will lead to the accumulation of senescent cells that will eventually promote cellular aging (2). Scientific evidence suggests senescence as a causal factor in many age-related diseases such as, arthritis, diabetes and cancer(9). mTOR is an evolutionarily conserved protein kinase signaling pathway that regulates cell growth, and is of great interest to understand how senescence affects aging(10). Although senescent cells do not replicate, they are metabolically functional and mTOR pathway has been shown to promote senescence(11). Therefore, a significant correlation exists between oxidative stress, cellular senescence and aging; inhibition of mTOR pathway was repeatedly shown to extend longevity in animal models. A pharmacological intervention that could decelerate the process of these cellular mechanisms will be of great advantage to improve health and life span.

Sulindac: Sulindac is an FDA-approved NSAID that has been used clinically for more than 30 years. Data from previous studies show that Sulindac protects the rat heart against ischemia/reperfusion (I/R) damage in a Langendorff model of cardiac ischemia by a preconditioning mechanism independent of it NSAID activity (5). In these earlier experiments, damage to the heart was measured both by lactate dehydrogenase (LDH) release and infarct size. Sulindac reduced LDH release by close to 60% during the ischemic period and 80% during reperfusion, and there was also a corresponding reduction in infarct size. We also showed that sulindac protects cardiomyocytes against the damage induced by hypoxia/reoxygenation treatment. Sulindac has also been reported to protect rats from I/R damage in an animal stroke model (13). As a model in vitro system for AMD, we have studied the effect of oxidative stress on retinal pigmented epithelial cells (RPE), which are known to be sensitive to oxidative stress.

These studies initially used the established ARPE19 cell line and the results showed that sulindac could protect RPE cells against chemical oxidative damage (Figure 1) (6).

Markers: Western blot is a laboratory technique used to detect a specific protein in a blood or tissue sample. The method involves using gel electrophoresis to separate the sample's proteins. The separated proteins are transferred out of the gel to the surface of a membrane. Once the proteins have been separated onto a membrane antibody marker are used in order to detect which proteins are present. Four markers were used in this experiment when performing the western blots: p-21, mTOR, p-AKT, and AKT. P-21 is a cyclin-dependent kinase inhibitor that triggers cell growth arrest that is associated with the senescence and damage response. p21 is transiently induced in the course of replicative senescence, reversible and irreversible forms of damage-induced growth arrest, and terminal differentiation of postmitotic cells; its induction is regulated through p53-dependent and -independent mechanisms (14). Ectopic overexpression of p21 leads to cell growth arrest in the $G_0$ phase; this arrest is accompanied by phenotypic markers of senescence in some or all cells. mTOR regulates cellular senescence through modulation of mitochondrial metabolism, autophagy and protein translation. mTOR homologs in many model organisms promote organismal aging through poorly characterized mechanisms. mTOR positively regulates protein synthesis and negatively regulates autophagy pathway [15]. Akt-deficiency causes resistance to replicative senescence, oxidative stress- or oncogenic Ras-induced premature senescence, and to reactive oxygen species (ROS)-mediated apoptosis. Akt activation induces premature senescence and sensitizes cells to ROS-mediated apoptosis by increasing intracellular ROS through increased oxygen consumption and by inhibiting the expression of ROS [16].

## Materials and Methods

Effect of sulindac on RPE cells exposed to TBHP: When testing for cell viability using RPE cells, RPE cells were plated at 10,000 cells per well using a F-12 DMEM media in a 96 well dish. The Experimental design has two groups: Group 1 was incubated at 37 degrees Celsius with Sulindac at 200µM for 24 hours and Group 2 was used a control group with no Sulindac. After the drug treatment was complete, the cells were exposed to an oxidative stressor (TBHP) for 2 hours with a concentration ranging from 0-300 µM. Once the treatments were complete, an MTS assay was used to look at the cell viability. The MTS assay is used to assess cell proliferation, cell viability and cytotoxicity. The MTS assay protocol is based on the reduction of the MTS tetrazolium compound by viable mammalian cells (and cells from other species) to generate a colored formazan dye that is soluble in cell culture media.

X-Beta Galactosidase Assay: For this experiment 6-well dishes were also used. In this part of the experiment 10,000 cells per well were used because counting and taking pictures of the cells became easier when using fewer cells. Because of the decrease in cell density, there had to be a change in the TBHP concentration and duration of exposure. In this part of the experiment a TBHP concentration of 100µM had to be used for 6 hours. A 200 µM concentration of TBHP for a 12-hour exposure killed the RPE cells being used. After plating 10,000 cells per well, the Sulindac treatment was initiated the next day to avoid an over confluency in the cells. After a 24-hour drug treatment using Sulindac, the cells were treated with TBHP at 100µM for 6 hours. Once this was complete, the cells were stained using the senescence assay kit. After being stained, the cells were viewed under a microscope and pictures were taken. After taking pictures, the cells were processed through a computer software called

image J in which the senescent cells were differentiated from the normal cells. The senescent cells developed a blue/green pigmentation while the normal cells stayed a normal gray color.

Optimization of sulindac concentration, TBHP concentration and exposure time to establish an in-vitro senescence model: RPE cells were originally plated at 30,000 cells per well using a 6-well dish. Once the cells were plated, they were left in the incubator at 37℃ with complete media to become confluent. Once the cells were confluent, they were treated with different concentrations of TBHP to induce senescence. The concentrations ranged from 100µM to 500µM. An X-Beta galactosidase assay was used to stain the senescent cells in the different conditions. Senescence-associated beta-galactosidase (SA-β-gal or SABG) is a hypothetical hydrolase enzyme that catalyzes the hydrolysis of β-galactosidase into monosaccharides only in senescent cells. Senescence-associated beta-galactosidase, along with p16Ink4A, is regarded to be a biomarker of cellular senescence. After the cells had been grown in the 6-well dishes and had been treated with TBHP the senescence assay could be used. The first step was rinsing the media from the dishes followed by fixing the cells with a G/F fixative mix (1500uL per well). The cells would then be incubated for 5 min at room temperature. Once this was complete the cells were rinsed twice with PBS. Then the staining solution was added and left in a $CO_2$ free incubator for 12-16 hours. The staining solution included 250µL of 200µM Potassium Ferricyanide, 250 µL of 200 µM Potassium Ferrocyanide, 100 µL of 200 µM $MgCl_2$, 250 µL of 6M NaCl, and 200 µL of 50mg/mL X Beta-gal in DMSO. It is important that the X-Gal and the solution as a whole does not get exposed to light.

It was important to make sure that there was an equal cell concentration among all the different conditions that were being tested. To look at the concentration and, more importantly the viability, of the cells, we performed a trypan blue staining assay which shows which cells are

alive based on whether they take in the blue dye or not. Because live cells have an intact cell membrane, trypan blue cannot penetrate the cell membrane of live cells and enter the cytoplasm. In a dead cell, trypan blue passes through the porous cell membrane and enters the cytoplasm.

Western blots were done to show the presence of p-21.. The first step in a western blotting procedure is to separate the macromolecules in a sample using gel electrophoresis. Subsequently, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Most commonly, the transferred protein is then probed with a combination of antibodies: one antibody specific to the protein of interest (primary antibody) and another antibody specific to the host species of the primary antibody (secondary antibody). Often the secondary antibody is complexed with an enzyme, which when combined with an appropriate substrate, will produce a detectable signal. Chromogenic substrates produce a precipitate on the membrane resulting in colorimetric changes visible to the eye. The most sensitive detection methods use a chemiluminescent substrate that produces light as a byproduct of the reaction with the enzyme conjugated to the antibody. The light output can be captured using film. However, digital imaging instruments based on charge-coupled device (CCD) cameras are becoming popular alternatives to film for capturing chemiluminescent signal. Alternatively, fluorescently tagged antibodies can be used, which require detection using an instrument capable of capturing the fluorescent signal. P-21 is activated in response to DNA damage caused by telomere attrition, oxidative stress, or oncogenic stress. This type of DNA damage leads to chronic activation of p-21 which leads to cellular senescence. During these studies, I performed the exact experiments in terms of cell culturing with the same drugs and oxidative stressors. After completing my drug

treatments, I harvested my cells using a lysis buffer with a protease inhibitor cocktail. After harvesting the cells, I measured the protein concentrations using the BCA method. The BCA protein assay is used for quantitation of total protein in a sample. The principle of this method is that proteins can reduce $Cu^{2+}$ to $Cu^{1+}$ in an alkaline solution (the biuret reaction) and result in a purple color formation by bicinchoninic acid. $Cu^{2+}$ is added to the sample and is reduced to $Cu^{1+}$ by protein. This step must be performed in basic solution with sodium potassium tartrate. This step is sometimes referred to as the biuret reaction and it forms light blue color that is very faint and difficult to quantify on its own. The three amino acids most involved in the reaction are cysteine, tyrosine, and tryptophan. The peptide backbone also contributes to the reaction, which makes this assay different from the coomassie protein detection method. Bicinchoninic acid is added to the sample to react with the $Cu^{1+}$ produced by the protein. This second step produces a purple reaction product that is much brighter than the blue color produced in step 1. Two molecules of BCA react with each $Cu^{1+}$. My conditions were control, cells treated with 200 µM TBHP, cells treated with 75uM Sulindac, and cells treated with 75uM Sulindac with 200uM TBHP. After quantifying the proteins through the BCA method, these samples were ran through a gel and then transferred them onto a membrane and the membrane was probed with appropriate primary and secondary antibody to analyze the expression of these proteins under the experimental conditions.

Results

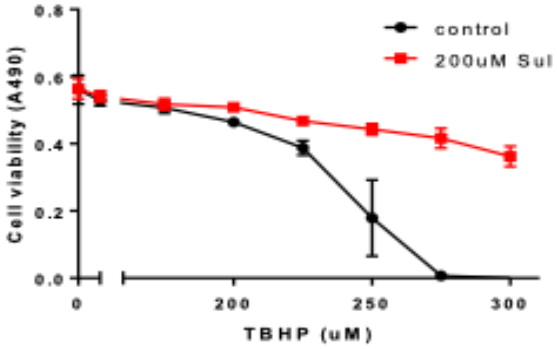



Figure 1: Sulindac protects RPE cells from oxidative damage induced by hydrogen peroxide

Figure 1 Effect of Sulindac on RPE Cells: This figure shows the protection that Sulindac gives to the RPE cells when they are exposed to an oxidative stressor like TBHP. The data shows a steady decrease in cell viability when the cells are exposed to TBHP. However, when the cells are pre-exposed to Sulindac there is an increase in cell viability compared to the TBHP treatment. An MTS assay was used on order to test the effect of TBHP on the cell viability of the RPE cells

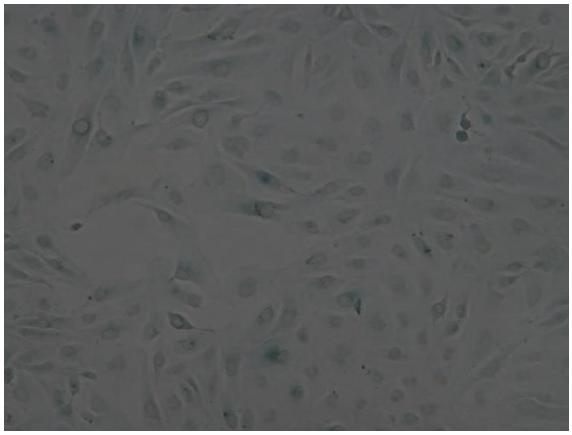


Figure 2 Sulindac and TBHP Treatment: This figure shows the effect of Sulindac on TBHP again using the X-Beta galactosidase assay. Sulindac had to be optimized during this stage since the TBHP was already optimized at a 200uM concentration. Sulindac concentrations started at 25uM and went up to 200uM. Concentrations from 25uM to 50uM had no effect on the oxidative stressor as senescence was still occurring based on the staining. At 75uM there was a consistent reduction in the number of senescent cells being produced. All the concentrations above 75uM gave an increased number of senescent cells in the wells.

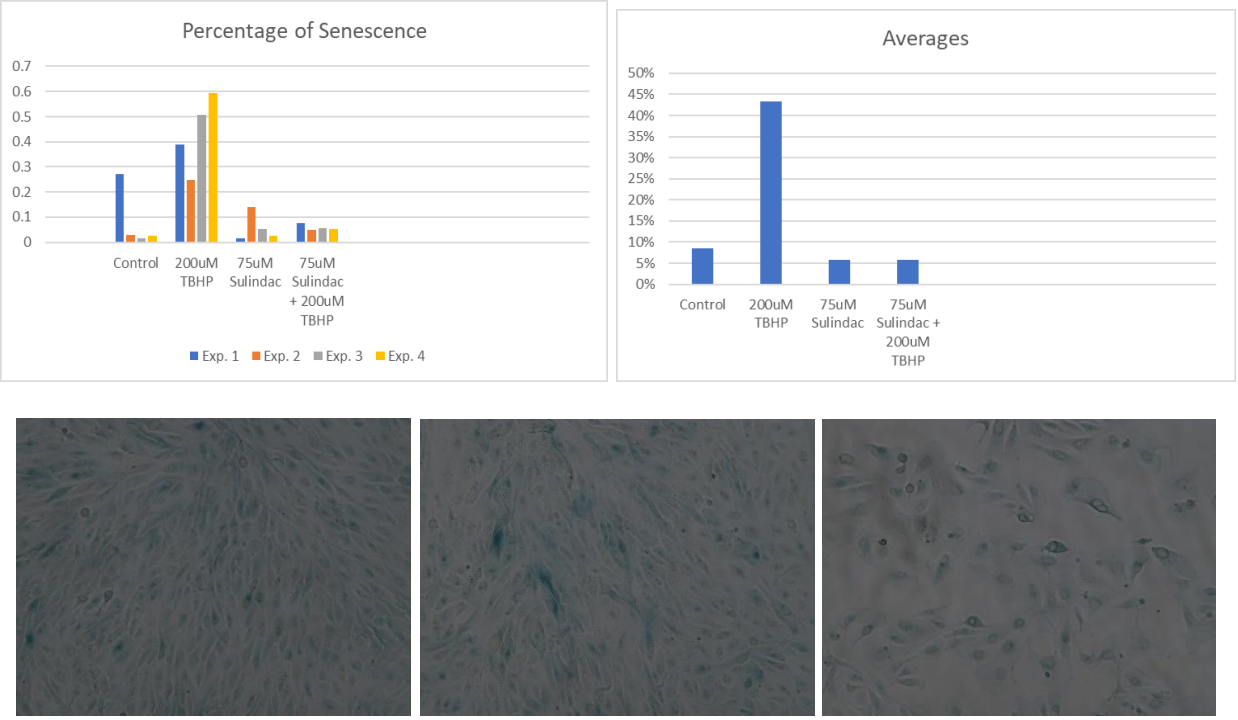
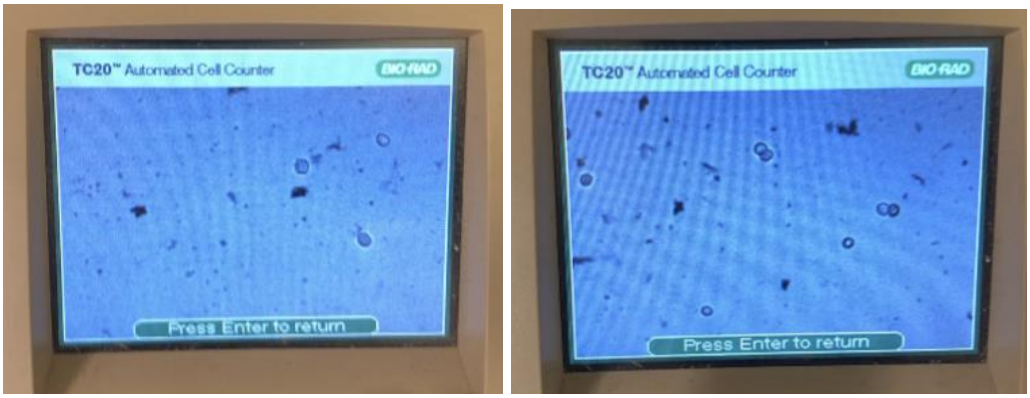





Figure 3 Percentage of Senescence in RPE Cells: This figure shows the optimization of TBHP doses when used on RPE cells. The RPE cells were grown in 6-well dishes and then left to incubate until they were confluent. The cells were then treated with TBHP starting from a concentration of 50uM and then moving up to 200uM concentration with increments of 50uM. In the beginning stages of the experiment, 200uM was shown to be the best concentration of the stressor in order to cause the cells to go into senescence. Time exposure ranged from 2 hours to 12 hours. Too low of a concentration did not force the cells into senescence and too great a concentration would kill the cells. The optimized time and concentration in order to induce senescence was 200uM at 12 hours. This was quantified and observed using an X-Beta galactosidase assay staining kit. Once the cells were stained, Image J was used in order to count the number of senescent cells verse normal cells.



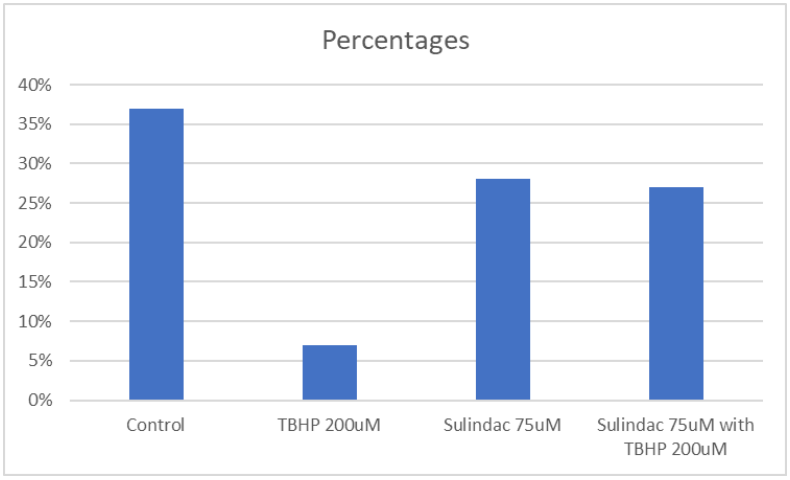




Figure 4 Trypan Blue Viability Assay: To make sure that the cell concentration was not decreasing due to the oxidative stressor or the drug treatment, a trypan blue viability assay was inducted. The assay concluded that there was still enough cell viability to continue the experiments with the same cell density and drug concentration. The blue staining was taken up by the membranes of dead cells whereas cells that were viable did not take in the dye. The main reason why this assay needed to be done is because at about halfway through the testing phases of Sulindac, our oxidative stressor (TBHP) was accidentally thrown away. Since the TBHP was thrown away the lab purchased more, but the new TBHP ended up killing the RPE cells most likely due to the potency of the drug. For approximately 4 months I had to work on reoptimizing the concentrations of TBHP in order to make sure that they did not kill my RPE cells but also

induced senescence in my cells. After about four months the reoptimized concentration of TBHP was 100uM instead of 200uM and a treatment for 6 hours instead of 12 hours.

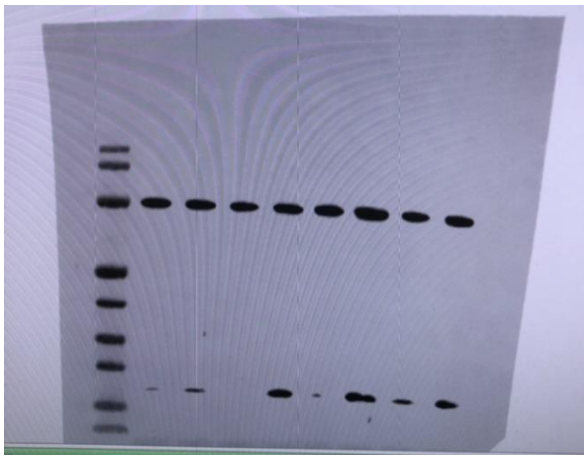
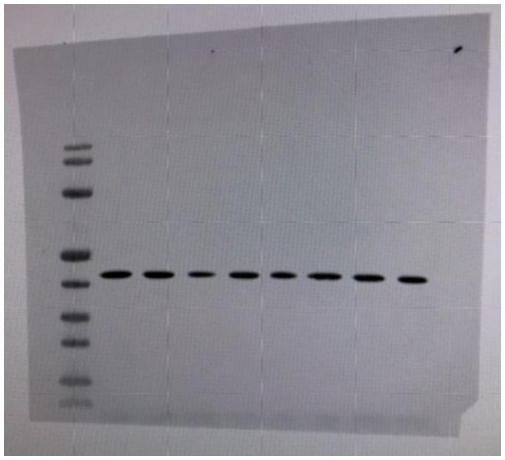
P21:  Actin: 

Figure 5 Western Blots of the RPE Cells: Multiple western blots were done in order to probe for p-21, pAKT, AKT, and mTOR. Although we did not get replicative data we found a greater expression of p-21 in cells that were treated with 200uM TBHP for a period of 12 hours.

## Discussion

Sulindac has been shown to have dual properties: it protects normal cells (like RPE cells) against oxidative damage but it also enhances killing of cancer cells in the presence of an oxidizing agent. Sulindac acts differentially by taking advantage of the metabolic properties of the cell. It has been shown that sulindac has properties that will make it an ideal drug to protect normal cells from oxidative damage and thereby delay the process of aging and age-related pathologies. The data from these experiments shows that there were a reduced number of senescent cells in the samples treated with sulindac compared to the control groups. These findings indicate that oxidative damage induces cell senescence, and sulindac can decrease cellular senescence induced by oxidative damage in RPE cells. Therefore, Sulindac can be a

potential therapeutic drug that can be used to mitigate senescence and thus slow down the progression of AMD. Not only can this drug be tested with RPE cells, but they can also be used to different types of cells in the body to slow down the process of aging and thus slowing down the progression of age-related diseases.

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