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5 Lipoxygenase's Role in Chondrogenesis of the ATDC5 Cell Line

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by

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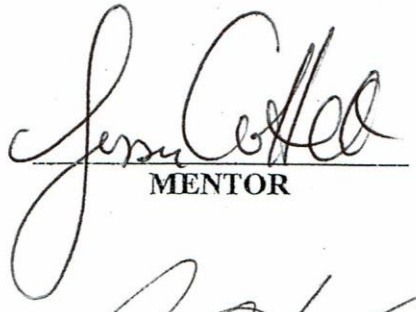
Seton Hall University
Department of Biological Sciences

APPROVED BY



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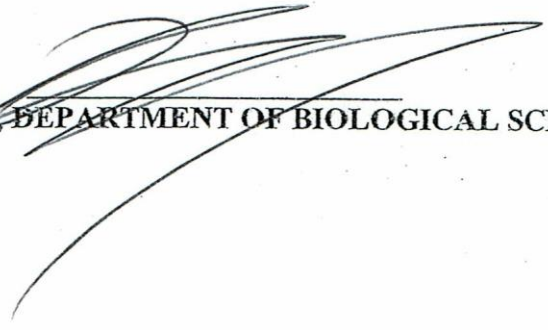

CHAIRPERSON, DEPARTMENT OF BIOLOGICAL SCIENCES

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Abstract

5-Lipoxygenase metabolizes arachidonic acid (AA) to generate leukotrienes (LTs). Leukotrienes are very potent inflammatory mediators that can have negative effects on bone healing and cartilage tissue generation. Recent studies have shown that decreasing 5-lipoxygenase (5-LO) activity during fracture healing accelerates endochondral ossification and decreases healing time in animal fracture models, however, the roles of 5-LO in chondrogenesis is not fully investigated. In this study, we tested the hypothesis that 5-LO inhibition will increase the process of chondrogenesis. The ABT-761 is an N-hydroxyurea analog structurally similar to zileuton, which is a 5-LO enzyme inhibitor. The ATDC5 cell line which is derived from teratocarcinoma cells of mice is a chondrogenic cell line. The ATDC5 cells were treated with ABT-761 and Insulin. Then, they were evaluated for cellular proliferation via colorimetric assay, 5-LO gene expression via Immunocytochemistry (ICC) and Immunoblotting (Western Blot), and chondrogenesis by measuring calcium deposition, proteoglycan synthesis via Alizarin Red stain and Alcian Blue stain respectively at specific time points 7 days, 14 days, 21 days and 28 days. Our data demonstrated that ATDC5 cells express a 5-LO protein which can be directly inhibited by ABT-761 treatment. At a concentration of 10 µM, ABT-761 treatment does not affect ATDC5 cellular proliferation, however, higher doses inhibit significantly. In term of chondrogenesis, in this study, we demonstrated that 1µM ABT-761 has no effect on chondrogenesis, neither increase nor decrease, in ATDC5 chondrogenic cell line. In contrast, concentrations of 10 µM and 100µM ABT-761 have negative effects on chondrogenesis through which ABT-761 promotes chondrocyte

apoptosis that may potentially lead to accelerating endochondral ossification or long bone formation in patients with a long bone fracture. However, ABT-761 may not be a clinical value to regenerate or promote chondrogenesis in patients suffering from osteoarthritis.

Key words: osteoarthritis, chondrocyte, chondrogenesis, ossification, ABT-761, ATDC5 cell.

Introduction

Cartilage biology

Cartilage is a strong, flexible and semi-rigid supporting connective tissue. Like all connective tissue, cartilage consists of cells called chondrocytes and extracellular matrix (ECM). Chondrocytes are highly specialized cells which are derived from mesenchymal stem cells and occupy from 1% to 5% of total cartilage tissue (Bhosale and Richardson 2008). They are metabolically active cells that proliferate and secrete ECM to maintain, sustain, and repair the cartilage (Akkiraju and Nohe 2015). Cartilaginous extracellular matrix is composed of 70-80% water, 10-25% collagen fibers which have high amounts of type II collagen, and 5-15% proteoglycans (PGs) which have a large composition of aggrecans (Horkay 2012) that contain non-collagenous protein and glycoproteins including glycosaminoglycan (GAG) and hyaluronic acid (HA) (Gao, Liu et al. 2014). Water and proteoglycans provide cartilage tissues compressive strength while the collagenous network is responsible for the tensile strength (Bautista, Park et al. 2016). The three-dimensional network of ECM is formed by the triple-helical collagen fibrils. The major cartilage PG is the bottle brush aggrecan molecule, which interacts with hyaluronic acid (HA) to form large proteoglycan aggregates (size 1-4 micrometers) or aggrecan-HA complexes. These complexes are embedded in the fibrous collagenous network (Horkay 2012) (***Figure 1***).

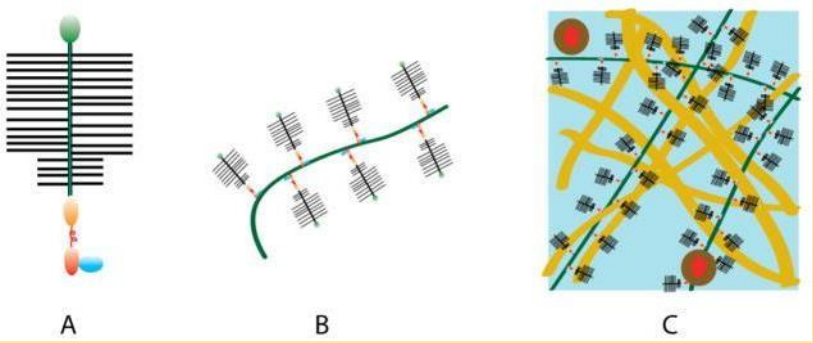

***Figure 1** shows the hierarchy of cartilage extracellular matrix organization. (A) Aggrecan bottlebrush. (B) Aggrecan-HA complex. (C) Aggrecan-HA complexes aggregates in the collagenous network (Horkay 2012)*

There are three types of cartilages, including hyaline cartilage, elastic cartilage, and fibrous cartilage. Elastic cartilage is found at the external ear, ear canal, epiglottis of the larynx. Fibrous cartilage is found in intervertebral disks of the vertebral column and menisci of the knee, forms the attachment of ligaments and tendons to bone. Hyaline cartilage comprises cartilage at synovial joint including shoulders, elbow, hips, sternum, and cartilage of nasal septum, larynx, trachea, and bronchi. Hyaline cartilage is also called articular cartilage and is 2-4 mm thick (Sophia Fox, Bedi et al. 2009). There are two important functions that articular cartilage performs. First, along with synovial fluid, articular cartilage provides virtually friction-free movement within the joint. Second, cartilage spreads the load in weight-bearing joints across the joint surface, which allows the underlying bones to absorb the shock and weight. Hence, articular cartilage functions require it to be elastic and to have high tensile strength. These characteristics are provided by ECM components, proteoglycans and Type II collagen, which are both produced by chondrocytes (Bautista, Park et al. 2016). Similar to bone tissue, cartilage

degrades and replaces its matrix constantly. Unlike bone tissue, however, cartilage is devoid of blood vessels, lymphatics and nerves, therefore, cartilage has very limited capability to heal and repair in a harsh biochemical condition (Sophia Fox, Bedi et al. 2009). Nutrition is supplied through simple diffusion of synovial fluid. Thus, the regeneration capacity of articular cartilage is very limited. As a result, injuries that do not penetrate the subchondral bone cannot self-repair in adults (Dehne, Karlsson et al. 2009).

Chondrogenesis and chondrocyte differentiation during development

Cartilage has an important function by serving as a template for long bone formation through endochondral ossification (Kronenberg 2003). Chondrogenesis is a complex process that leads to establishments of cartilage. During embryogenesis, cartilage is the first skeletal tissue to be formed that leads to the formation of all mature cartilage and long bones, except flat bones (Quintana, zur Nieden et al. 2009). Flat bone is formed through intramembranous ossification, while long bone formation is through endochondral ossification. The process of cartilage formation starts when mesenchymal stem cells (MSCs) condense to form a dense cell mass. Then MSCs proliferate and differentiate into chondroblasts. Some of these cells give rise to mature chondrocytes. If mature chondrocytes undergo endochondral ossification, or long bone formation, cartilage is vascularized, ECM becomes degraded, hypertrophic chondrocyte become apoptotic, and osteoblasts invade the free space within the tissue (Hoffman, Weston et al. 2003), (Mackie, Ahmed et al. 2008). (***Figure 2***). As chondrogenesis plays important roles

in the formation of both mature cartilages and bones, any errors occurring during this crucial process can affect cartilage formation and long bone formation.

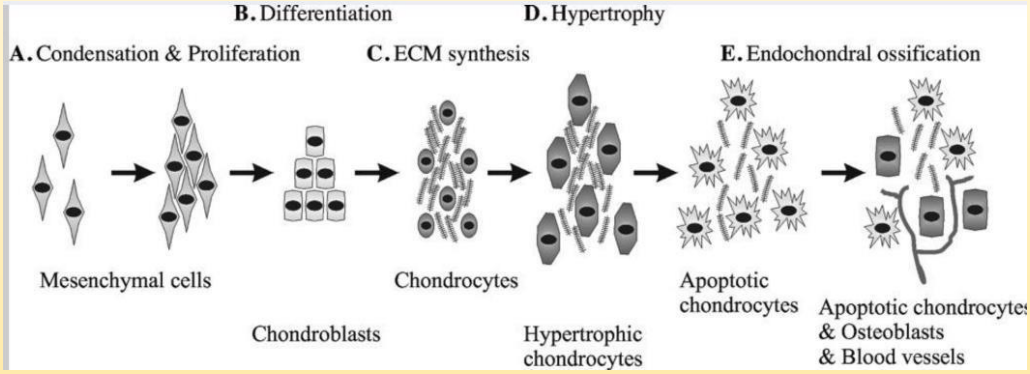

Figure 2 *shows the schematic of chondrogenesis and endochondral ossification Chondrogenesis can be divided into five main stages, each stage is the occurrence of a specific event. (A) Condensation and proliferation of MSCs, (B) Differentiation of MSCs into chondroblasts, (C) Chondroblast become chondrocytes (D) Mature chondrocytes become hypertrophy, (E) if cartilage tissue undergoes endochondral ossification hypertrophic chondrocytes become apoptotic, cartilage is vascularized, and osteoblasts invade the tissue (Quintana, zur Nieden et al. 2009)*

Arachidonic acid and leukotriene biosynthesis

Arachidonic acid (AA) is the precursor of prostaglandins and leukotrienes, which are lipid signaling molecules. In mammals, Cyclooxygenase 1 and cyclooxygenase 2 (COX 1 and COX 2) are the key enzymes in prostaglandins biosynthesis, while 5-lipoxygenases (5-LO), is a key enzyme in leukotriene biosynthesis. Despite having approximately 60% identical primary structures and performing almost the same catalytic function (Simmons, Botting et al. 2004), COX 1 is constitutively expressed in most tissues, while COX 2 is induced (Tanabe and Tohnai 2002). COX2 metabolizes AA to generate cyclo-endoperoxides including prostaglandin H2 (PGH2), which is subsequently converted into PGD2, PGE2, PGF2, PGI2, and thromboxane A2 (TXA2). In leukotriene biosynthesis, 5-LO converts AA into 5 hydroxy-peroxy-eicosatetraenoic acid (5HPETE), which is then converted into intermediate leukotriene A4 (LTA4), then into leukotriene B4 (LTB4) by enzyme leukotriene A4 hydrolase (LTA4H), leukotriene C4 (LTC40, leukotriene D4 (LTD4), leukotriene E4 (LTE4) by enzyme LTC4 synthase (LTC4S) (O'Connor, Manigrasso et al. 2014). (***Figure 3***).

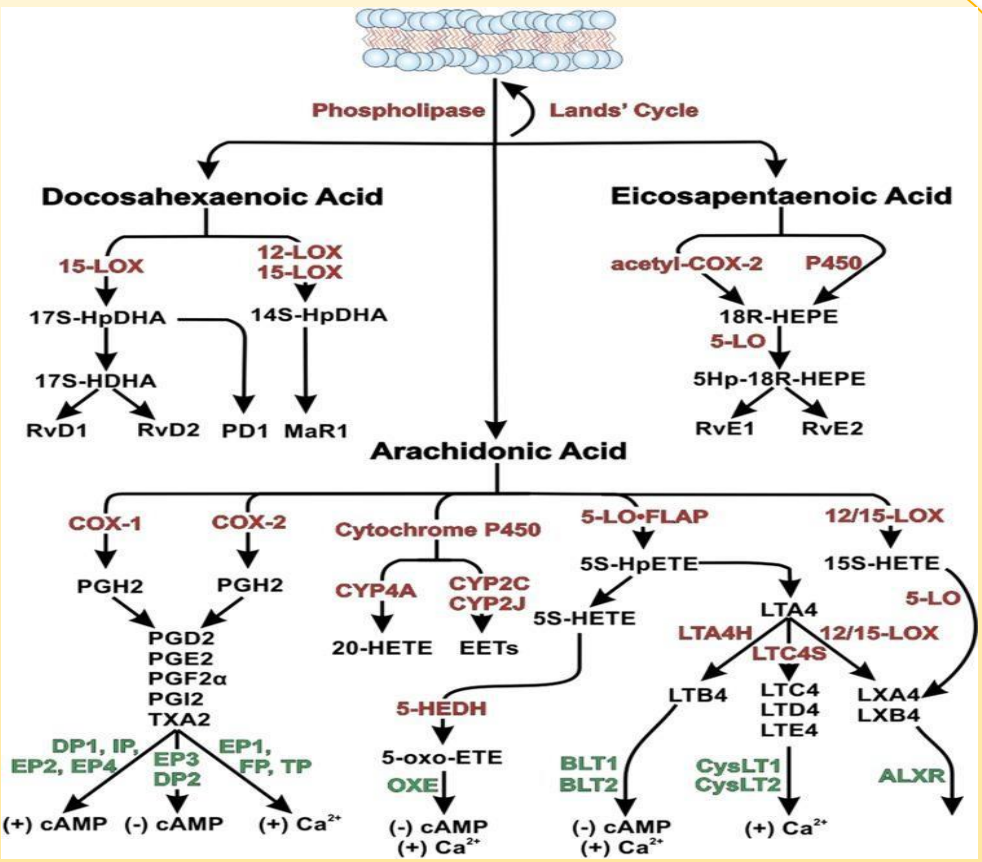

Figure 3: Prostaglandin and Leukotriene biosynthesis (O'Connor, Manigrasso et al. 2014) *shows schematic of pathway for synthesis and signaling of arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid-derived lipid mediators. The diagram summarizes some of the synthetic pathways for lipid mediator synthesis. Lipid mediators and synthetic intermediates and active products are shown in black text. Enzyme activities are shown in red text. Receptors are shown in green text. Known effects of receptor activation on intracellular cyclic adenosis monophosphate or calcium levels are shown at the bottom.*

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Prostaglandins and leukotrienes biosynthesis have been showed to be important in the process of chondrogenesis and endochondral ossification or long bone formation. Leukotrienes are very potent inflammatory mediators that can have negative effects on long bone healing and cartilage tissue generation. Recent studies have shown that decreasing 5-LO activity during long bone fracture can accelerate endochondral ossification, decrease healing time, and increase osteogenesis in animal models Other studies also proved that leukotriene antagonism, using montelukast sodium which is leukotriene type 1 receptor antagonist and zileuton which is a 5-LO enzyme inhibitor, can enhance fracture repair on mice modal by increasing chondrocyte proliferation and early endochondral bone formation (Wixted, Fanning et al. 2009). Licofelone (ML-3000), a dual inhibitor of 5-LO and COX, has been shown to reduce the level of cartilage chondrocyte death in vivo in experimental dog osteoarthritis (Boileau, Martel-Pelletier et al. 2002). these experimental findings indicate that pharmacological manipulation of the arachidonic acid pathway could be a means to accelerate or enhance chondrogenesis. In fact, there are a great number of compounds targeting 5-LO or its cofactor, 5-Lipoxygenase-Activating factor (FLAP), which have been used to treat chronic obstructive pulmonary diseases ((Poff and Balazy 2004), (Carter, Young et al. 1991, Evans, Ferguson et al. 2008),(Muller-Peddinghaus, Fruchtmann et al. 1993). In addition, compounds targeting leukotriene receptors or prostaglandin receptors have been used to treat ophthalmic conditions, asthma, and other medical conditions (Jones, Labelle et al. 1995), (Alexander, Miller et al. 2002).

Relation to public health and purpose of the thesis

As chondrogenesis is the important process in the formation of mature cartilage and endochondral ossification (Quintana, zur Nieden et al. 2009), pharmacological methods to promote chondrogenesis would be of clinical value for osteoarthritis and long bone fractures. Osteoarthritis (OA) is a clinical syndrome that is characterized by cartilage degradation that leads to joint failure, in addition to stiffening of joints, and functional limitation (Akkiraju and Nohe 2015). With OA, the cartilage within the joint pathologically lost hyaline or articular cartilage itself, subchondral sclerosis development, osteophyte formation, and meniscus and bone marrow degradation (Joseph, Baum et al. 2012), ligamentous laxity, and subchondral bone changes (Bennell, Hunter et al. 2012). Joints affected by OA are peripheral joints which include large joints such as the knees, hips, and small joints such as hand joints. As these joints are important to carry out daily activities, the pain from these joints can reduce joint functions and effect on patients' quality of life. Not only does OA impact the individual, OA can also have large impact on society, affecting almost 27 million Americans or 12.1% of the adult population. It is estimated that the United States spends $185 billion in management of OA annually (Neogi 2013). Thus, methods that could regenerate or promote chondrogenesis would be of substantial benefit to patients and would reduce the societal burden caused by OA. Usually, patients suffering from OA receive both pharmacological (such as celecoxib, rofecoxib, which are nonsteroidal anti-inflammatory drugs or NSAIDS, acetaminophen, capsaicin cream) and non-pharmacological treatments (such as exercise, aerobic or strengthening or water-based, arthroplasty). Although, these treatment options can relieve

symptoms or improve functionality temporarily, pharmacological options are known to have adverse side effects such as gastrointestinal (GI) toxicity of NSAIDs and increased risk of myocardial associated with rofecoxib (Zhang, Moskowitz et al. 2007).

In this study, we hypothesize that direct pharmacological inhibition of 5-LO would accelerate and enhance chondrogenesis and may contribute to the improved bone healing effects observed in the rodent fracture models where 5-LO expression was inhibited (Cottrell, Keshav et al. 2013). Our hypothesis was tested with the use of ATDC5 chondroprogenitor cells treated with a direct 5-LO inhibitor, ABT-761. The ATDC5 cell line is derived from the mouse teratocarcinoma cells and has been characterized as a chondrogenic cell line which goes through a sequential process analogous to chondrocyte differentiation. ATDC5 cell line was chosen over a mice model because these cells are easy to handle and their differentiation into chondrocytes is easily detectable and have been used to assess for chondrogenesis as previous studies (Choi, Nepal et al. 2011), (Altaf, Hering et al. 2006). ABT-761 is a N-hydroxyurea analog which functions as a direct 5-LO enzyme inhibitor. ABT-761 exhibits potent and selective inhibition of leukotriene formation. As calcium and proteoglycan deposition occur during chondrogenesis, quantification of these matrix components is used to assess chondrogenesis.

Methodology

Cell culture

The ATDC5 cell line was utilized and stored at -80 C when not in use. Cells were cultured under 5% CO2 in growth media, which consisted of Dulbecco's Modified Eagle's Medium (DMEM)/Hams F-12 50/50 (Corning ®, Manassas, Virginia, USA) Mix supplemented with 5% fetal bovine serum (FBS), 1% L-Glutamine, 1% Penicillin/Streptomycin, 1% Selenium, and 1% Transferrin at 37°C. Cells were checked periodically (every 2-3 days) using a Leica DMi1 FOV20 inverted microscope (Leica, Chicago, IL, USA) to determine confluence, the possibility of contamination, and cell death. When the cells reached 100% of confluence, the experimental treatments were conducted as described below.

ABT-761 treatment

ATDC5 cells were treated with increasing doses of 5-LO inhibitor, ABT-761 (Abbott, Abbott Park, IL, USA), (1 µM, 10 µM, 100 µM, 1000 µM) in triplicate over specific time points (24 and 48 hours, and 1, 2, 3, 4 weeks) and/or with Insulin (Invitrogen, Waltham, MS, USA) (INS) at increasing doses (0.1% and 1%).

Cellular proliferation

ATDC5 cells were seeded in a 96-well plate at a density of 8000 cells per well and allowed to adhere for 24 hours at 37°C in a CO2 incubator. After 24 hours of incubation, culture media were replaced with the 12 different treatment groups (media, media and 0.1% INS, media and 1% INS, media and 10 µM ABT, media and 10 µM and 0.1% INS, media and 10 µM ABT and 1% INS, media and 100 µM ABT, media and 0.1% INS and 100 µM ABT, media and 1% INS and 100 µM ABT, media and 1000 µM ABT, media and 0.1% INS and 1000 µM ABT, media and 1% INS and 1000 µM ABT). Then the cells were incubated for 24 hours at 37°C in a CO2 incubator. Subsequently, 10 µL of yellow MTT solution (5 mg/ml in Phosphate-Buffered Saline (PBS), 1X, without Calcium and Magnesium, Corning®, Manassas, Virginia, USA) was added to each well and the 96-well plate was incubated for 4 hours at 37°C in a CO2 incubator. Then the mixture of treatment and MTT solution was aspirated from each well. Next, the formed formazan crystals were solubilized by adding 100 µL of Dimethyl Sulfoxide (DMSO), (Sigma-Aldrich ®, USA), per well. Each plate was covered with aluminum foil and incubated at room temperature for 2 hours while shaking. Finally, the intensity of the dissolved intracellular purple formazan crystal was quantified by measuring the absorbance at a wavelength of 570 nm using spectrophotometry SpectraMax M5. In order to quantify % of cellular proliferation, the absorbance value reading of the blank must be subtracted from all samples. Absorbance reading from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. The formula was shown in ***Figure 4.*** If the absorbance values are greater

than the control, this indicates increased cell proliferation, but if the absorbance values are lower than the control this suggests cell death or inhibition of cellular proliferation.

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

Figure 4: formula for determine cellular proliferation

Immunocytochemistry

ATDC cells were seeded in quadruplet in an 8-chamber polystyrene vessel tissue culture treated glass slide (Falcon Corning ®, Big Flats, New York, USA) at a density of 80,000 cells/300µL per well and allowed to adhere for 24 hours at 37°C in a CO2 incubator. After 24 hours of incubation, the culture medium was replaced with 4 different treatment groups (media, media and 10 µM ABT, media and 1% INS, media and 1% INS and 10 µM ABT). At timepoints of 24 and 48 hours, the immunocytochemistry was conducted. First, the media in the chambers was aspirated, then the chamber was washed one time with PBS. Next, the cells were fixed in 5 minutes with -20°C methanol. Then, the fixative -20°C methanol was aspirated, and the chamber was rinsed 3 times with ice-cold PBS. After that, the chambers were incubated with 300 µL blocking reagent, SuperBlock™ Blocking Buffer in PBS (Thermo Scientific, Rockford, Illinois, USA) to block unspecific binding for 1 hour at room temperature. Subsequently, the blocking reagent was aspirated, and the chambers were incubated with primary antibodies, 5-

Lipoxygenase (5-LO) mouse monoclonal antibody, (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) (sc-136195), at 1:25 dilution at 4°C overnight. On the following day, the chamber was washed 3 times with PBS), 1X, without Calcium and Magnesium, (Corning®, Manassas, Virginia, USA), for 5 minutes and gentle shaking. Then the chamber was incubated with secondary antibodies, mouse-IgG kappa BP-HRP which is a purified recombinant mouse IgG kappa light chain binding protein conjugated to horseradish peroxidase (HRP), (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) (sc-516102), at 1:25 dilution for 2 hours. After that, the chambers were washed 3 times with PBS for 5 minutes while gently shaking. Next, the chambers were incubated with Hydrogen peroxide 30% solution and DAB Substrate System, (VWR Company, Solon, Ohio, USA) for 2 to 5 minutes following the manufacturer's instructions. Then the chambers were washed 2 times with diH2O. After that, the safe removal fixture kit was used to remove the chamber out of the slide. Finally, the slides were dehydrated through alcohol and xylenes as follows: soak in 90% ethanol twice for 3 minutes each, then 100 % twice for 3 minutes each. Immediately, 1-2 drops of permanent Acrytol Mounting Media, (Leica Biosystems, Buffalo Grove, IL, USA) were added on the slides and covered with glass coverslips. Each slide was observed by Olympus BX53 Microscope at 200X magnification, and Osteomeasure software was used to quantify the DAB staining procedure and document the images.

Protein Isolation

First, adherent ATDC5 cells treated with six treatment groups (0.1% INS, 1% INS, 0.1%INS and 10 µM ABT, 1% INS and 10 µM ABT, 10 µM ABT, and media only) were harvested with Mammalian Protein Extraction Buffer (Thermo Fischer Scientific, Handouver Park, IL, USA) while on ice. Six different treatment groups were aspirated from the plates, rinsed with cold 1x HBSS, then 1X HBSS was aspirated. Mammalian Protein Extraction Buffer was added to each well and the plate was placed onto a shaker for ten minutes to coat cells evenly while on ice. Next, a cell scraper was used to remove adherent cells off the plate, assist in cell lysis, and protein extraction. The protein lysates collected from each well were stored in the -20°C freezer until needed for further analysis.

Western Blotting

Triplicate sets of frozen protein samples in Mammalian Protein Extraction Buffer were thawed on ice until completely defrosted to prevent protein degradation. In separate microtubes, protein sample and 2X SDS with 2-Mercaptoethanol (2-Me) were combined in a 1:1 ratio and centrifuged at 4 C. Protein mixture was heated in a dry bath at 70°C for 10 minutes to denature the protein. A 4-12% NuPAGE Bis-Tri gel (Thermo Fischer Scientific, Handover Park, IL, USA) was run using NuPAGE MOPS Running Buffer solution (Thermo Fischer Scientific, Handover Park, IL, USA) and run for 50 minutes at 200V. In preparation for gel transfer, the transfer apparatus and four pieces of filter paper

were soaked in a transfer buffer solution with 10-20% methanol; polyvinylidene fluoride (PVDF) membrane was also soaked for one minute in methanol only and rinsed in the NuPAGE transfer buffer solution. After the running gel was complete, proteins affixed to the gel were transferred to the polyvinylidene difluoride (PVDF) membrane following the manufacturer's directions. Following transfer, PVDF membrane was placed in SuperBlock solution (ThermoScience, Rockford, IL) and rocked at room temperature for 1 hour.

After 1 hour, the PVDF membrane was rinsed 3 times in 1x Transfer Buffer Saline with 0.1% TWEEN (TBS-T) for 5 minutes, then placed in primary antibody solution, and slowly shaken overnight in 4°C refrigerator. The primary antibody solution consisted of 5-Lipoxygenase (5-LO) mouse monoclonal antibody, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA (sc-136195, and 0.1% SuperBlock™ Blocking Buffer in PBS (Thermo Scientific , Rockford, Illinois, USA). Internal control for 5-LO protein expression is beta-actin gene. After overnight incubation, the PVDF membrane was rinsed 3 times in 1X TBS-T for 5 minutes of each rinse, then placed in secondary antibody solution, and rocked at room temperature for 1 hour. The second antibody solution consisted of anti-mouse IgG-conjugated antibody, TBS-T and 0.1% SuperBlock. The membranes were rinsed twice in 1X TBS-T and once in 1X TBS to remove any remaining secondary antibodies, then placed in Pierce ® Enhanced Chemiluminescence (ECL) Western Blotting substrate (Thermo Fischer, Handover Park, IL, USA) to view under UltraViolet light using FluorChem E system (ProteinSimple, San Jose, CA, USA). Then the image result was analyzed by using Alpha View software in which the analysis

tool and multiplex band analysis tool were used, the 5-LO protein expression was normalized using beta-actin gene.

Alcian Blue Staining

In order to detect proteoglycan deposition, Alcian Blue Assay was used. First, the ATDC5 cells were plated in triplicate in 24 well-plate at a density of 50,000 cells/500 µL per well. After 2 days of incubation for confluence at 37ºC in a CO2 incubator, the growth media was replaced with 8 different treatment groups including media, media and 1 µM ABT, media and 10 µM ABT, media and 100 µM ABT, media and 1% INS, media and 1% INS and 1 µM ABT, media and 1% INS and 10 µM ABT, media and 1% INS and 100 µM ABT, and the time points of 1, 2, 3, 4 weeks, the cells were harvested. At each time point, on the day 1 of the Assay, the 24-well plates were rinsed twice with PBS then fixed with 100% cold methanol for 5 minutes. Next, the plates were stained with 0.1% Alcian Blue in 0.1 M HCl overnight at room temperature. The following day, the 24-well plates were rinsed 3 times with HBSS. Then, proteoglycan was extracted with 6 M Guanidine-HCl overnight. Lastly, the proteoglycan concentration was quantified by measuring the absorbance at a wavelength of 595 nm using a spectrophotometer.

Alizarin Red Staining

In order to detect calcium deposition, Alizarin Red Assay was used. Like Alcian Blue Stain protocol, the ATDC5 cells were plated in triplicate in 24 well-plate at a density of 50,000 cells/ 500 µL per well. After 2 days of incubation for confluence at 37°C in a CO2 incubator, the growth media was replaced with 8 different treatment groups including media, media and 1 µM ABT, media and 10 µM ABT, media and 100 µM ABT, media and 1% INS, media and 1% INS and 1 µM ABT, media and 1% INS and 10 µM ABT, media and 1% INS and 100 µM ABT. At timepoints of 1, 2, 3, 4 weeks, the cells were harvested. The 24-well plates were rinsed 2 times with PBS then fixed with 10% (v/v) formaldehyde at room temperature for 15 minutes. Next, the plates were rinsed 2 times with diH2O, and 1 mL of 40 mM Alizarin Red dye solution was pipetted into each well. Then, the 24-well plates were incubated for 20 minutes at room temperature. After that, the Alizarin Red dye was aspirated, the plates were washed 4 times with diH2O while shaking for 5 minutes of each wash. To facilitate the removal of any remaining liquid, the plates were left at an angle for 2 minutes and re-aspirated. After that, the plates were stored in -20ºC freezer. On the following day, 800 µL of 10% (v/v) of Acetic acid was added to each well, then the plates were incubated at room temperature for 30 minutes while shaking. Using a cell scraper, the monolayer was scraped from the plate and transferred with 10% (v/v) acetic acid to clean centrifuge tubes. The centrifuge tubes were then vortexed briefly for 30 seconds, then warmed for 10 minutes at 85°C. Afterward, the centrifuge tubes were placed on ice for 5 minutes then centrifuged at 13,000 rpm for 15 minutes. Next, 500 µL of supernatant was transferred to

new centrifuge tubes. Then, 200 µL of 10% (v/v) ammonium hydroxide was added to the supernatant. Finally, 150 µL of supernatant was pipetted in triplicate into a 96-well plate, then the calcium deposition was quantified by measuring the absorbance at a wavelength of 405 nm using a spectrophotometer.

Results

The results of the MTT assay shows that 10µM ABT does not affect cellular proliferation compared to the control. However, at higher concentrations, ABT significantly inhibits cellular proliferation ***(Figure 5***).

The MTT assay result shows that 10 µM ABT (Rx3) does not affect cellular proliferation (113%, $p > 0.05$) compared to the controls, including media only (Rx1), 0.1% INS (Rx2), 1% INS (Rx9). However, at higher concentrations of 1000 µM, including Rx3 vs Rx7 (10 µM ABT vs 1000 µM ABT), Rx3 vs Rx8 (10 µM ABT vs 0.1%INS & 1000 µM), Rx 3 vs Rx12 (10 µM ABT vs 1%INS & 1000 µM), ABT significantly inhibits cellular proliferation, which decreases 51.5%, 52,8%, 52% respectively, p-value < 0.05. At concentrations of 100 µM, on the other hand, ABT does not inhibit cellular proliferation, Rx3 vs Rx5 (10 µM ABT vs 100 µM ABT), p> 0.05. Therefore, the concentration of ABT was capped in at 100 µM and one more concentration of ABT, which was 1 µM, was added for the following experiments to elucidate the effects of ABT on chondrogenesis. It is also noticed that there is no difference in the concentration of insulin at 0.1% and 1.0% regarding cellular proliferation in the controls group (Rx2 vs Rx9), as well as in the presence of 10 µM ABT (Rx4 vs Rx10) ***(Figure 5***), (***Table1, 2)***.

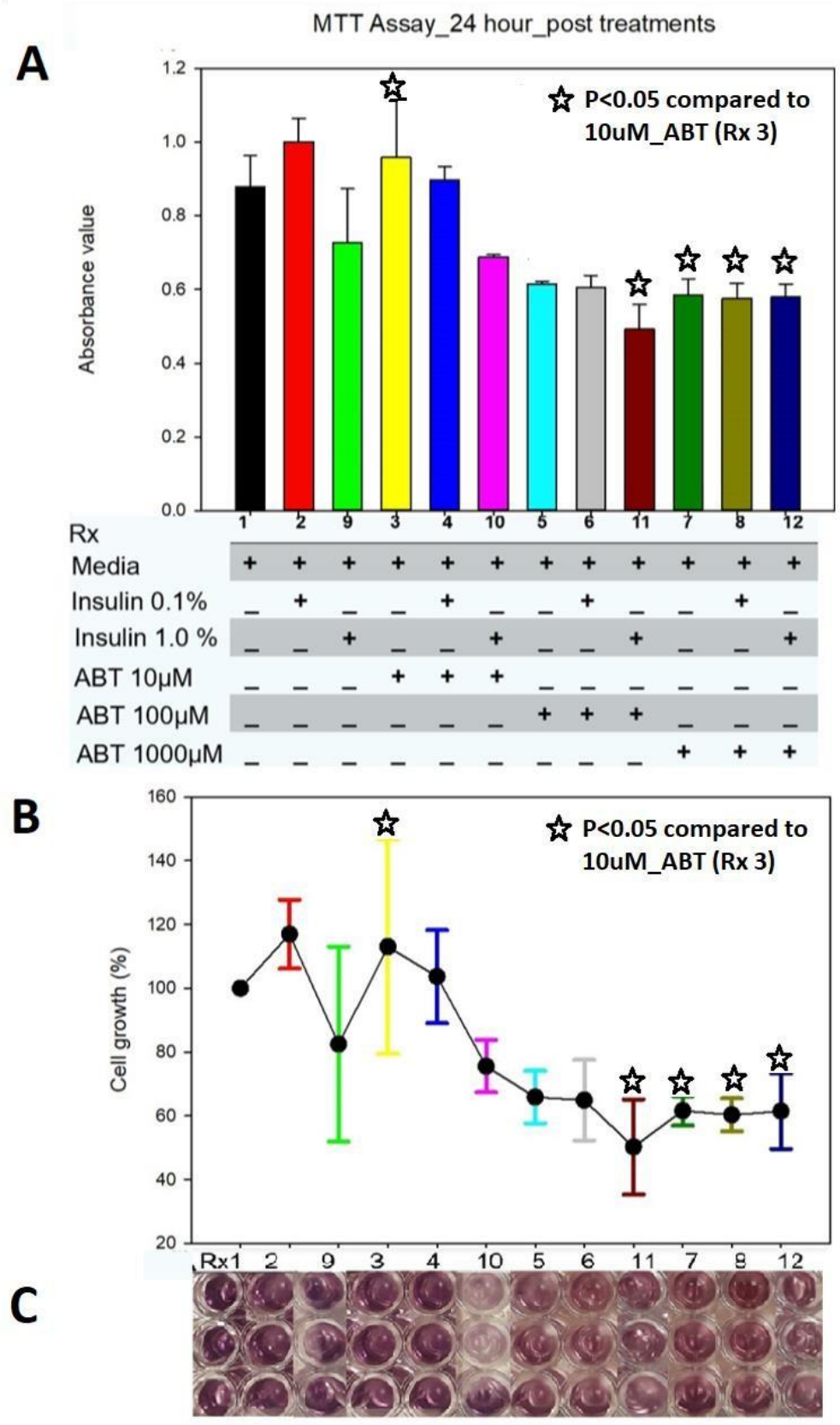

A
MTT Assay_24 hour_post treatments
Absorbance value
1.2
1.0
0.8
0.6
0.4
0.2
0.0
☆ P<0.05 compared to 10uM_ABT (Rx 3)
Rx 1 2 9 3 4 10 5 6 11 7 8 12
Media + + + + + + + + + + + +
Insulin 0.1% _ + _ _ + _ _ + _ _ + _
Insulin 1.0 % _ _ + _ _ + _ _ + _ _ +
ABT 10μM _ _ _ + + + _ _ _ _ _ _
ABT 100μM _ _ _ _ _ _ + + + _ _ _
ABT 1000μM _ _ _ _ _ _ _ _ _ + + +
B
Cell growth (%)
160
140
120
100
80
60
40
20
☆ P<0.05 compared to 10uM_ABT (Rx 3)
Rx 1 2 9 3 4 10 5 6 11 7 8 12
C

Figure 5: Cellular proliferation of ATDC-5 cells post ABT-treatment. ATDC-5 cells treated with 10 µM, 100 µM, 1000 µM ABT in combination with 0.1% and 1% insulin for 24 hours (Rx3,4,10,5,6,11,7,8,12_treatment groups).
Control groups included cells treated in media only, and media in combination with 0.1 % and 1% insulin (Rx1,2,9).
(A) The absorbance value of ATDC5 cell in each treatment group at 24 hours-post treatment.
(B) Cellular proliferation values calculated for ATDC5 cells in each treatment group at 24 hours-post treatment.
(C) Image of wells for each ATDC5 cell treatment group after completion of MTT assay. Error bars represented Mean +/- SD. Photographed by the author, Tien My Tran, at Seton Hall University, South Orange, New Jersey, USA, in May 2019.
(*) denoted statistically significant differences between treatment groups where appropriate; corresponding p-value were noted.

One Way Analysis of Variance			
Group Name		**Mean**	**Std Dev**
	SEM		
RX 2	(0.1%INS)	116.983	10.808
	6.240		
RX 9	(1.0%INS)	82.516	30.559
	17.643		
RX 3	(10 uM ABT)	113.068	33.693
	19.453		
RX 4	(0.1%INS &10 uM ABT)	103.685	14.563
	8.408		
RX 10	(1.0%INS & 10 uM ABT)	75.554	8.232
	4.753		
RX 5	(100 uM ABT)	65.866	8.160
	4.711		
RX 6	(0.1%INS & 100 uM ABT)	64.916	12.587
	7.267		
RX 11	(1%INS & 100 uM ABT)	50.257	14.903
	8.604		
RX 7	(1000 uM ABT)	61.615	4.660
	2.691		
RX 8	(0.1%INS & 1000 uM ABT)	60.303	5.171
	2.985		
RX 12	(1%INS & 1000 uM ABT)	61.475	11.846
	6.840		

Table 1: cellular proliferation percentage in each treatment group compare to control (Rx1) which is 100%

Comparison	Diff of Means (%)	t	P	P<0.050
RX 2 vs. RX 11 (0.1%INS vs 1%INS & 100 uM ABT)	66.725	5.083	0.002	Yes
RX 3 vs. RX 11 (10 uM ABT vs 1%INS & 100 uM ABT)	62.811	4.785	0.005	Yes
RX 2 vs. RX 8 (0.1%INS vs 0.1%INS & 1000 uM ABT)	56.680	4.318	0.015	Yes
RX 2 vs. RX 12 (0.1%INS vs 1%INS & 1000 uM ABT)	55.507	4.229	0.018	Yes
RX 2 vs. RX 7 (0.1%INS vs 1000 uM ABT)	55.368	4.218	0.019	Yes
RX 4 vs. RX 11 (10 uM ABT & 0.1%INS vs 1%INS & 100 uM ABT)	53.427	4.070	0.027	Yes
RX 3 vs. RX 8 (10 uM ABT vs 0.1%INS & 1000 uM ABT)	52.766	4.020	0.030	Yes
RX 2 vs. RX 6 (0.1%INS vs 0.1%INS & 100 uM ABT)	52.067	3.966	0.033	Yes
RX 3 vs. RX 12 (10 uM ABT vs 1%INS & 1000 uM ABT)	51.593	3.930	0.036	Yes
RX 3 vs. RX 7 (10 uM ABT vs 1000 uM ABT)	51.454	3.920	0.036	Yes
RX 2 vs. RX 5 (0.1%INS vs 100 uM ABT)	51.117	3.894	0.038	Yes
RX 1 vs. RX 11 (media only vs 1%INS & 100 uM ABT)	49.743	3.789	0.048	Yes
RX 3 vs. RX 1 (10 uM ABT vs media only)	13.068	0.996	1.000	No
RX 2 vs. RX 3 (0.1%INS vs 10 uM ABT)	3.914	0.298	1.000	No
RX 3 vs. RX 9 (10 uM ABT vs 1.0% INS)	30.552	2.327	0.669	No
RX 3 vs. RX 5 (10 uM ABT vs 100 uM ABT)	47.203	3.596	0.074	No
RX 2 vs. RX 9 (0.1%INS vs 1.0% INS)	34.466	2.626	0.458	No
RX 4 vs. RX 10 (10 uM ABT & 0.1%INS vs 10 uM ABT & 1%INS)	28.131	2.143	0.799	No

Table 2: statistical analysis for the cellular proliferation in each treatment group of the MTT assay in ***Figure 1***.

The Immunocytochemistry shows ATDC5 cells do express 5-Lipoxygenase (5-LO) protein in growth and differentiation phase, and the 5-LO protein can be inhibited in the presence of the inhibitor, ABT-761 ***(Figure 6,7***). The results of Immunocytochemistry show that at both 24 and 48-hour post-treatment, there is an increase in 5-LO protein expression in both only media group (Rx1) and 1%INS group (Rx9). However, the 5-LO protein expression decreases in the presence of 10 µM ABT (Rx3_only 10 µM ABT, and Rx 10_1%INS+10 µM ABT).

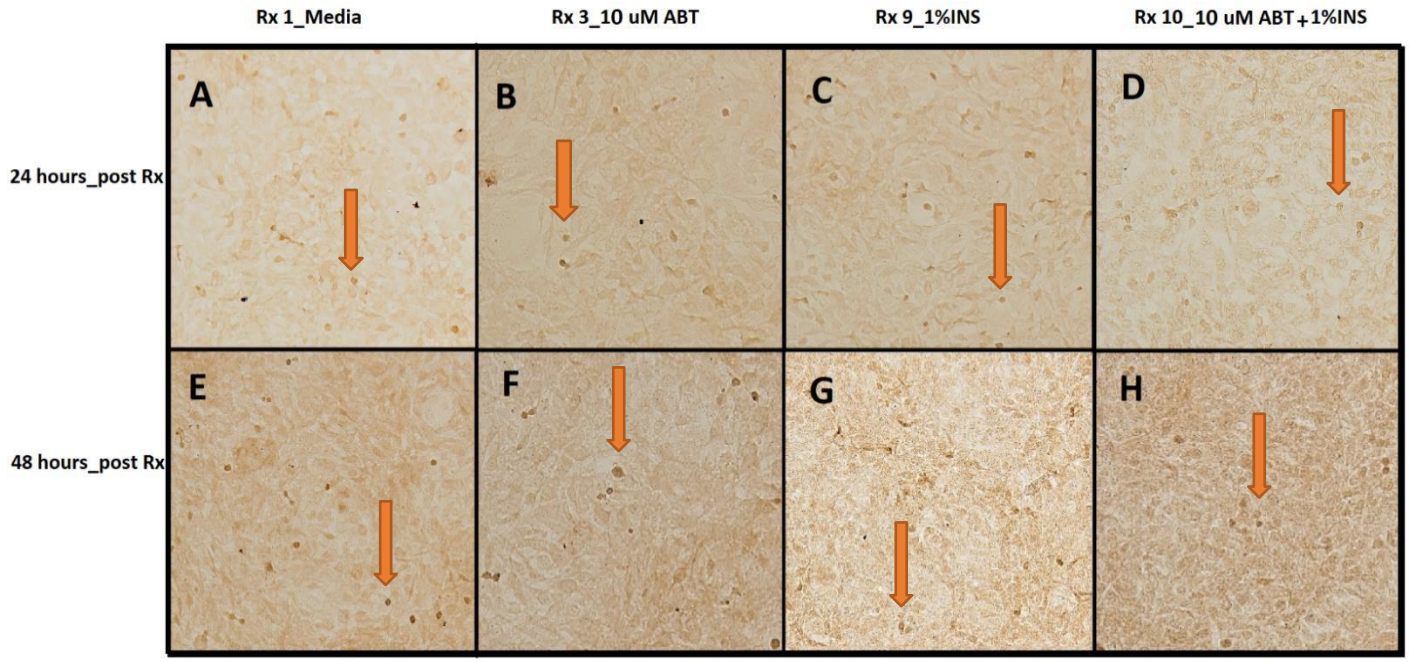


Figure 6: Immunocytochemistry of ABT-treated ATDC5 cells. ATDC5 cells were plated at 50% and allowed to reach confluency, then the media was replaced with 4 treatment groups: Rx1 (just Media), Rx3 (10µM ABT), Rx9 (1%INS), Rx10 (10µM ABT + 1% INS). Subsequently, the Immunocytochemistry was conducted at two time points: 24 hours-post treatments (A, B, C, D), and 48 hours-post treatments (E, F, G, H). ATDC5 cells stained for 5-LO protein using the primary antibody, 5-Lipoxygenase (5-LO) mouse monoclonal antibody, (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) (sc-136195) and then DAB substrate. Finally, 5-LO protein expression can be visualized as brown spots, which are denoted by orange arrows. Photographed by the author, Tien My Tran, at Rutgers University, Newark, New Jersey, USA, in May 2019.

	Rx1 _ Media	Rx3_1uM ABT	Rx9_1% INS	Rx10_1uM ABT + 1%INS
24 hours post Rx	74	45	96	41
	85	37	93	37
	89	52	104	44
48 hours post Rx	93	48	107	67
	96	37	100	63
	99	48	97	67

Table 3: Brown spot counting data using an Olympus BX53 Microscope at 200X magnification, and Osteomeasure software

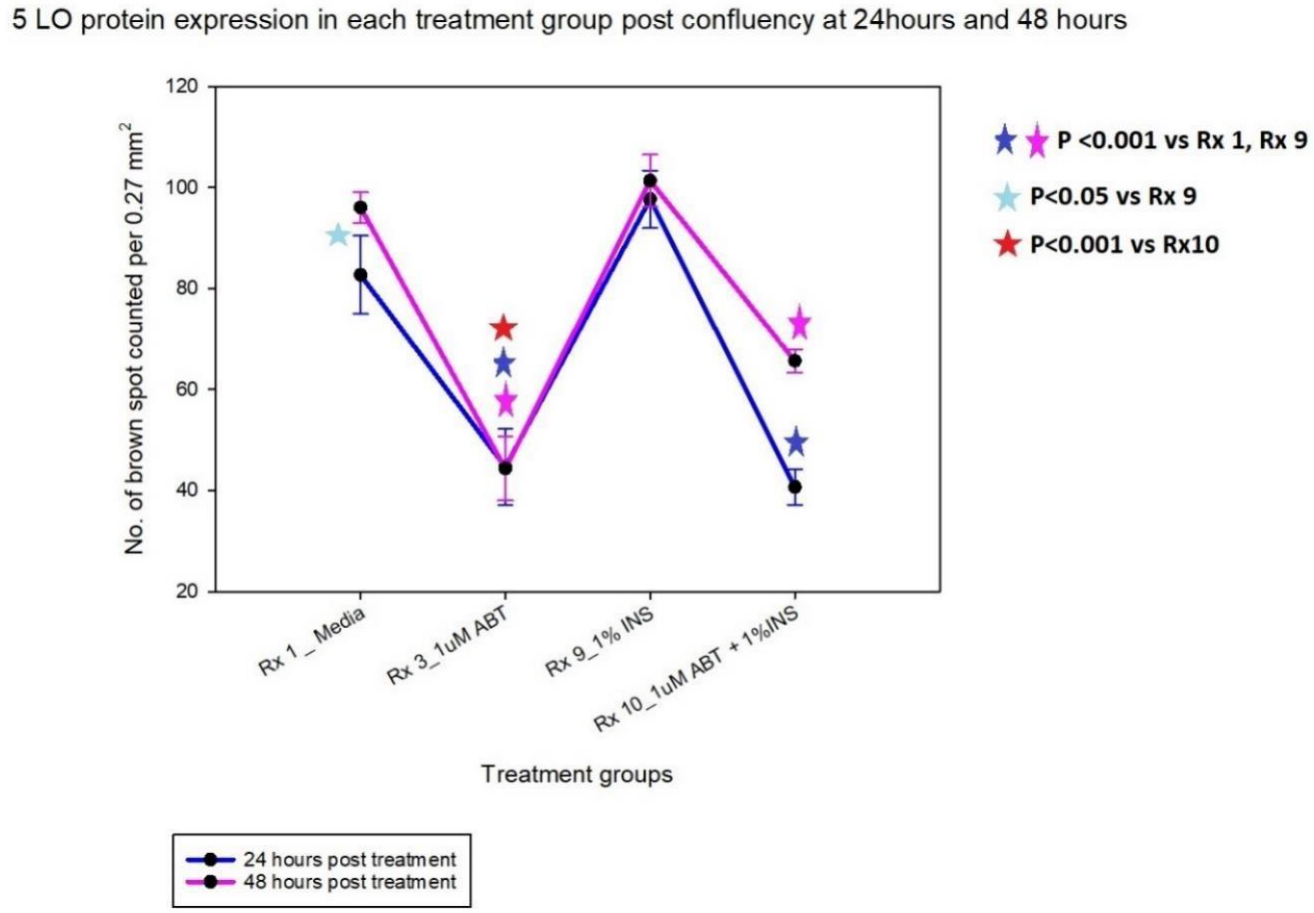


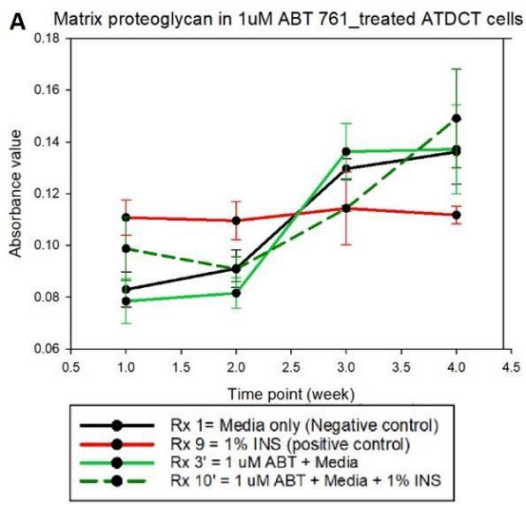
Figure 7: Number of 5-LO protein expression differences among 4 treatment groups: Rx1_only Media, Rx3_1 µM of ABT, Rx9_1% INS, Rx10_1µM ABT + 1%INS. At both time points at 24 and 48 hours post-treatment, 5-LO protein expression decreases in both treatment groups Rx3 (10 µM ABT) and Rx10 (1% INS+10 µM ABT) compared to the Rx1 (media only) and Rx9 (1.0% INS) ($p<0.05$), which are denoted by pink and blue starts. At timepoint of 24 hours post-treatment, 5-LO protein expression increases in Rx9 (1.0% INS) versus Rx1 (only Media), which is denoted by light blue stars. At time point of 48 hours, 5-LO protein expression increases in Rx10 (1% INS+10 µM ABT) versus Rx3 (10 µM ABT), which is denoted by red star. When compared to 48 hours post-treatment, 5-LO protein expression in Rx 1 (only media) and Rx 10 (1% INS+10 µM ABT) increase in 24 hours post treatment. In contrast, there are no differences in 5-LO protein expression in Rx3 (10 µM ABT) and Rx9 (1.0% INS) in both time points.

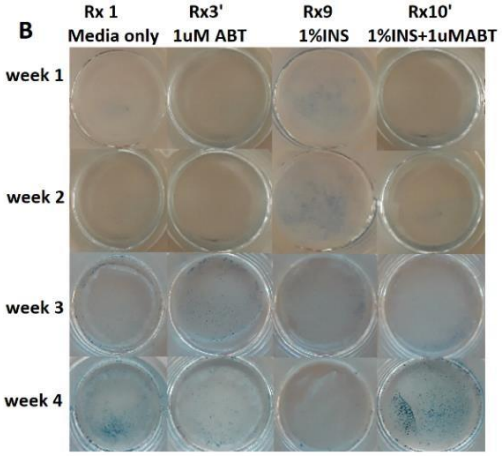
Comparison	Diff of Means	t	P
P<0.050			
24 hours post treatment			
(Rx9 vs Rx10) 1.0% INS vs 1 µM ABT + 1.0% INS Yes	57.000	10.992	<0.001
(Rx9 vs Rx3) 1.0% INS vs 1 µM ABT Yes	53.000	10.221	<0.001
(Rx1 vs Rx10) Media only vs 1 µM ABT + 1.0% INS Yes	42.000	8.100	<0.001
(Rx1 vs Rx3) Media only vs 1 µM ABT Yes	38.000	7.328	<0.001
(Rx9 vs Rx1) 1.0% INS vs Media only Yes	15.000	2.893	0.040
(Rx3 vs Rx10) 1 µM ABT vs 1 µM ABT + 1.0% INS No	4.000	0.771	0.463
48 hours post treatment			
(Rx9 vs Rx3) 1.0% INS vs 1 µM ABT Yes	57.000	15.513	<0.001
(Rx1 vs Rx3) Media only vs 1 µM ABT Yes	51.667	14.062	<0.001
(Rx9 vs Rx10) 1.0% INS vs 1 µM ABT + 1.0% INS Yes	35.667	9.707	<0.001
(Rx1 vs Rx10) Media only vs 1 µM ABT + 1.0% INS Yes	30.333	8.256	<0.001
(Rx10 vs Rx 3) 1 µM ABT + 1.0% INS vs 1 µM ABT Yes	21.333	5.806	<0.001
(Rx9 vs Rx1) 1.0% INS vs Media only No	5.333	1.452	0.185
24 hours vs 48 hours post treatment			
Rx1 Media only Yes	13.333	3.502	0.008
Rx 10 1 µM ABT + 1.0% INS Yes	25.000	6.565	<0.001
Rx3 1 µM ABT No	0.333	0.0655	0.949
Rx9 1.0% INS No	3.667	0.721	0.742

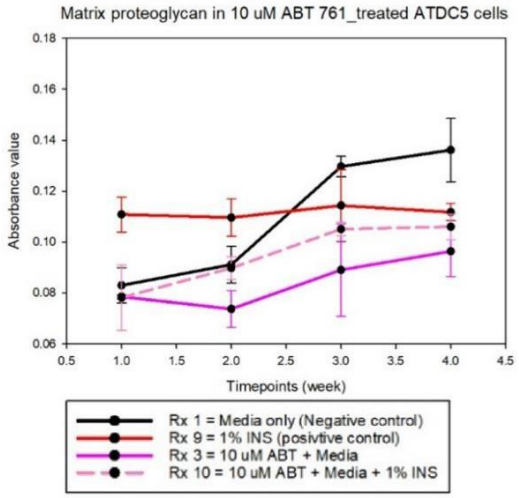
Table 4*:** statistical analysis for 5-LO protein expression in 4 treatment groups in ***Figure 8

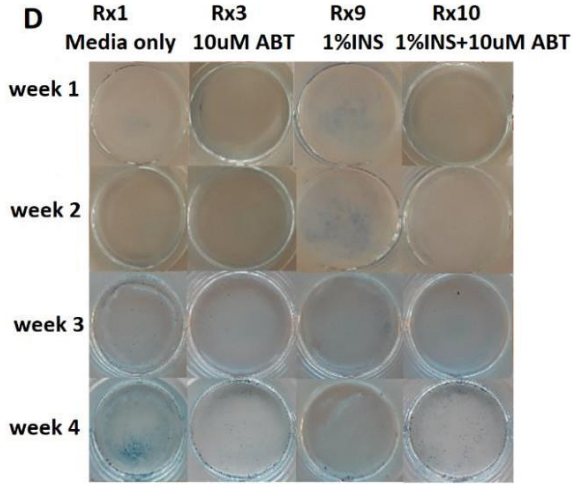
The Alcian Blue stain showed that treatment with ABT-761 has negative effect on matrix proteoglycan deposition (***Figure 8***) At week 1, the proteoglycan deposition of at treatment groups (Rx3'_1μM ABT, Rx3_10μM ABT, Rx5_100μM ABT, Rx10_1%INS+10μM ABT, Rx11_1%INS+100μM ABT) is lower compared to the positive control (Rx9_1%INS) ($p<0.05$), except treatment group Rx10'_1%INS+1μM ABT. Once again, at week 2, the proteoglycan production at all treatment groups including Rx10' is lower than positive control Rx9 ($p<0.05$)(***Figure 9***). However, Rx3 (10μM ABT) and Rx11 (1%INS+100μM ABT) is lower than negative control Rx1 (only Media) ($p<0.05$)(***Figure 9, E, F, C, D***). Especially, Rx 3'(only 1μM ABT) and Rx10'(1%INS+1μM ABT) are higher than Rx3, Rx5, Rx11 at which there are a higher concentration of ABT ($p<0.05$). Thus, from week 2, there is a possibility of higher concentrations of ABT which might decrease proteoglycan production has emerged. At week 3, as there is no statistically significant difference between proteoglycan production in all treatment groups compared to positive control Rx9 (1%INS) ($p>0.05$), in other words, proteoglycan production in all treatment groups starts increasing compared to decreasing in week 1, and week 2. Also, Rx3' (1μM ABT) shows higher proteoglycan deposition versus Rx3 (10μM ABT), Rx5 (100μM ABT), Rx10 (1%INS+10μM ABT), Rx11 (1%INS+100μM ABT). This further strengthens the possibility that higher concentrations of ABT can inhibit proteoglycan production. Finally, at week 4, once again, Rx3'(1μM ABT) and Rx10'(1%INS+1μM ABT) strongly show higher proteoglycan deposition versus Rx3 (10μM ABT), Rx5 (100μM ABT), Rx10 (1%INS+10μM ABT), Rx11 (1%INS+100μM ABT) ($p<0.01$). Thus, the Alcian blue

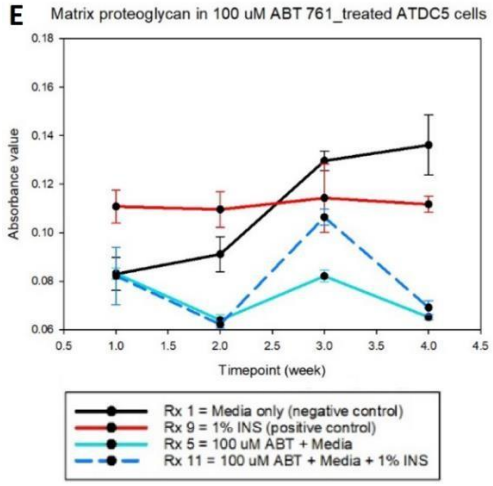
staining assay result showed that at a concentration of 1μM, ABT-761 has no effect on proteoglycan deposition of ATDC5 chondrogenic cell line, however, at higher concentrations, including 10μM and 100μM, ABT-761 has negative effects on proteoglycan deposition. Alizarin Red Stain also shows that treatment with ABT-761 also has negative effects on calcium deposition of ATDC5 chondrogenic cell line.


A
Matrix proteoglycan in 1uM ABT 761_treated ATDCT cells
Absorbance value
Time point (week)
Rx 1= Media only (Negative control)
Rx 9 = 1% INS (positive control)
Rx 3' = 1 uM ABT + Media
Rx 10' = 1 uM ABT + Media + 1% INS


B
Rx 1
Media only
Rx3'
1uM ABT
Rx9
1%INS
Rx10'
1%INS+1uMABT
week 1
week 2
week 3
week 4


C
Matrix proteoglycan in 10 uM ABT 761_treated ATDC5 cells
Absorbance value
Timepoints (week)
Rx 1 = Media only (Negative control)
Rx 9 = 1% INS (posivtive control)
Rx 3 = 10 uM ABT + Media
Rx 10 = 10 uM ABT + Media + 1% INS


D
Rx1
Media only
Rx3
10uM ABT
Rx9
1%INS
Rx10
1%INS+10uM ABT
week 1
week 2
week 3
week 4


E
Matrix proteoglycan in 100 uM ABT 761_treated ATDC5 cells
Absorbance value
Timepoint (week)
Rx 1 = Media only (negative control)
Rx 9 = 1% INS (positive control)
Rx 5 = 100 uM ABT + Media
Rx 11 = 100 uM ABT + Media + 1% INS

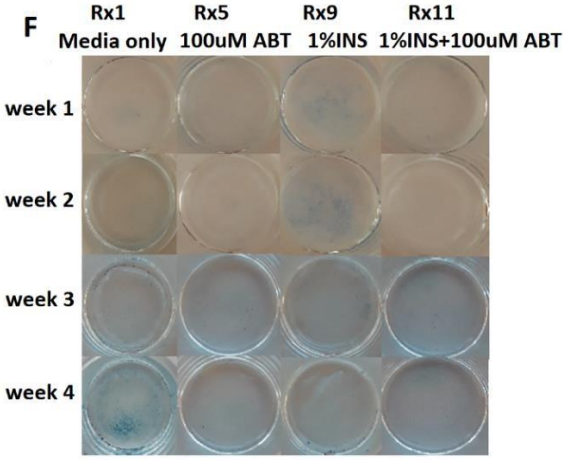

F
Rx1
Media only
Rx5
100uM ABT
Rx9
1%INS
Rx11
1%INS+100uM ABT
week 1
week 2
week 3
week 4

Figure 8: Matrix proteoglycan production in ABT-treated ATDC5 cells overtime. (A) Quantification of 1uM ABT Treatment groups (B) Images of 1uM ABT Treatment Groups (C) Quantification of 10uM ABT Treatment groups (D) Images of 10uM ABT Treatment Groups (E) Quantification of 100uM ABT Treatment groups (F) Images of 10uM ABT Treatment Group. Photographed by the author, Tien My Tran, at Seton Hall University, South Orange, New Jersey, USA, in May 2019.

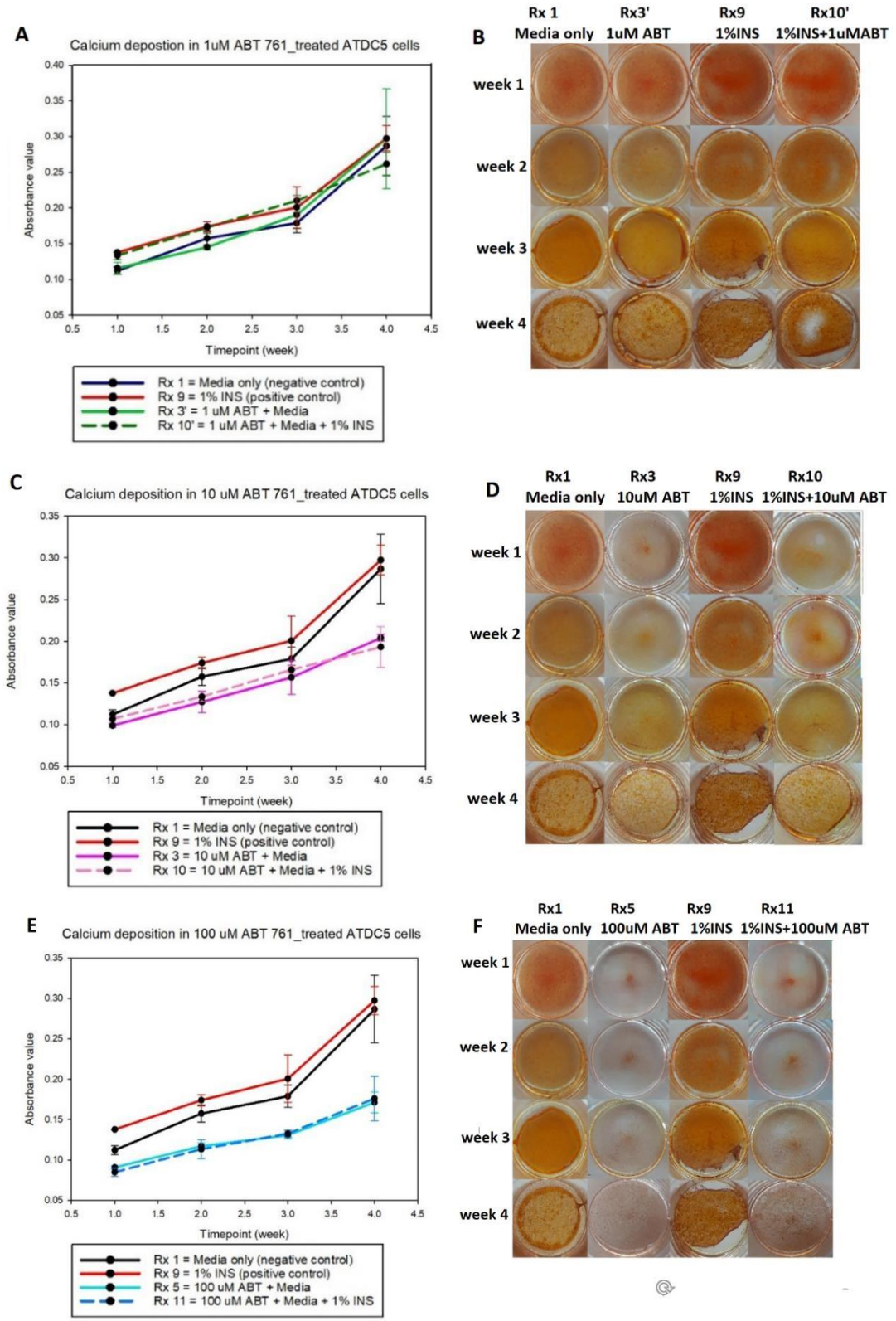

A
Calcium depostion in 1uM ABT 761_treated ATDC5 cells
Absorbance value
Timepoint (week)
Rx 1 = Media only (negative control)
Rx 9 = 1% INS (positive control)
Rx 3' = 1 uM ABT + Media
Rx 10' = 1 uM ABT + Media + 1% INS
B
Rx 1 Media only
Rx3' 1uM ABT
Rx9 1%INS
Rx10' 1%INS+1uMABT
week 1
week 2
week 3
week 4
C
Calcium deposition in 10 uM ABT 761_treated ATDC5 cells
Absorbance value
Timepoint (week)
Rx 1 = Media only (negative control)
Rx 9 = 1% INS (positive control)
Rx 3 = 10 uM ABT + Media
Rx 10 = 10 uM ABT + Media + 1% INS
D
Rx1 Media only
Rx3 10uM ABT
Rx9 1%INS
Rx10 1%INS+10uM ABT
week 1
week 2
week 3
week 4
E
Calcium deposition in 100 uM ABT 761_treated ATDC5 cells
Absorbance value
Timepoint (week)
Rx 1 = Media only (negative control)
Rx 9 = 1% INS (positive control)
Rx 5 = 100 uM ABT + Media
Rx 11 = 100 uM ABT + Media + 1% INS
F
Rx1 Media only
Rx5 100uM ABT
Rx9 1%INS
Rx11 1%INS+100uM ABT
week 1
week 2
week 3
week 4

Figure 9: Matrix Calcium Deposition in ABT-treated ATDC5 cells overtime. (A) Quantification of 1uM ABT Treatment groups (B) Images of 1uM ABT Treatment Groups (C) Quantification of 10uM ABT Treatment groups (D) Images of 10uM ABT Treatment Groups (E) Quantification of 100uM ABT Treatment groups (F) Images of 10uM ABT Treatment Groups. Photographed by the author, Tien My Tran, at Seton Hall University, South Orange, New Jersey, USA, in May 2019 .

Discussion

ABT-761, a new potent 5-Lipoxygenase inhibitor which effectively inhibits leukotriene formation has been evaluated both in vitro and in vivo for the treatment of leukotriene-dependent human diseases. In animal studies, the compound has shown it potentials in different disease models, including potently attenuates bronchoconstriction and pulmonary inflammation in rodent (Bell, Harris et al. 1997), or inhibit eosinophil influx into lungs of Brown Norway rats (Namovic, Walsh et al. 1996), or attenuates cerebral vasospasm in rabbit model of subarachnoid hemorrhage (Barbosa, Arthur et al. 2001). In fact, ABT-761 is under development for the treatment of asthma in both adults and children (Reid 2001),(Wong, Kearns et al. 1998). Although, there are studies demonstrated that pharmacological inhibition of 5-Lipoxygenase can accelerate and enhances fracture healing or bone formation (Cottrell and O'Connor 2009), (Cottrell, Keshav et al. 2013), (Saul, Weber et al. 2019). there are no direct studies focusing on the effects of ABT-761 on chondrogenesis. In this study, through the MTT assay, we found that ABT-761 at a concentration of 10 µM has no effect on cellular proliferation compared to the controls ($p>0.05$) (***Figure 5***). However, at higher concentrations, including 100 µM and 1000µM, ABT-761 significantly inhibits cellular proliferation, which decreases to 63% and 52% respectively, ($p<0.05$) (***Figure 5, Table 2***). In addition, we found that insulin concentration does not make any difference in cellular proliferation in the presence of ABT ($p>0.05$), (***Figure 5, Table 2***). Our data is similar to Steinhibler et al, who demonstrated that many 5-LO inhibitors have anti-proliferative effects. Therefore, moving forward from the MTT assay, we omitted the concentration of 1000

μM, and only used the two concentration of ABT-761 at 10 μM,100 μM, and added one new concentration of 1 μM to elucidate the effects of ABT-761.

In this study, using immunocytochemistry and immunoblotting, we showed that ATDC5 cells, which derived from mouse teratocarcinoma chondrogenic cell-like, do express 5 Lipoxygenase protein, in both proliferation and differentiation phases. 5-Lipoxygenase is a key enzyme in the metabolism of arachidonic acid to leukotriene. 5-lipoxygenase expression has been shown in various tissue, such as human brain with traumatic injury and astrocytoma, which is a form of brain tumor, has increased expression of 5-LO that was demonstrated by immunohistochemistry (Zhang, Zhang et al. 2006). In addition, using immunofluorescence and immunohistochemistry, 5-Lipoxygenase (5-LO) and 15-Lipoxygenase-1 (15-LO-1) have been demonstrated to be present in rheumatoid arthritis and osteoarthritis synovium, with 5-LO being mostly expressed in-lining and sub-lining macrophages, neutrophils and mast cells, and 15-LO-1 mainly in lining macrophages, fibroblasts, and sub-lining endothelial cells (Gheorghe, Korotkova et al. 2009). Leukotriene B4 (LTB4) is a potent chemoattractant associated with the development of osteoarthritis (OA), its receptors BLT1 and BLT2 have found in human synovium, subchondral bone as well as in cartilage cells or chondrocytes (Hansen, Indrevik et al. 2015). In this study, using immunocytochemistry and immunoblotting, we show that ATDC5 cells, which derived from mouse teratocarcinoma chondrogenic cell-like, do express 5 Lipoxygenase protein, in both proliferation and differentiation phases, in treatments with media only (Rx1) and 1%INS (Rx9) ($p<0.01$). Nevertheless, the 5-LO

protein expression is decreased in the presence of the inhibitor, ABT-761 (Rx3_10 μM ABT and Rx10_ 1%INS+10 μM ABT)($p<0.001$) (***Figures 6,7,8***).

In vertebrates, longitudinal bone growth occurs by a process called endochondral ossification, which takes place in the growth plates of long bones. Chondrocytes secrete cartilage matrix, proliferate and differentiate into hypertrophic cells during this process, resulting in progressive widening of the growth plate ((De Luca, Uyeda et al. 2000). There are many factors that can influence the longitudinal growth of bone. Nutrition, hormonal, and mechanical factors can all have effects on cell proliferation, hypertrophy, death and bone formation. In this study, we demonstrated that at a concentration of 1 μM ABT-761 (Rx3'), there is no increase nor decrease on proteoglycan deposition as well as calcium deposition compared to controls (Rx1_Media only and Rx9_1%INS) ($p<0.05$). Moreover, we strongly found that at higher concentrations of 10 μM (Rx3) and 100 μM of ABT-761 (Rx5), ABT-761 adversely inhibits proteoglycan production or calcium deposition ($p<0.001$) (***Figures 9.10***). In other words, these high concentrations of ABT-761 have negative effects on chondrogenesis as chondrogenesis is characterized by calcium deposition and proteoglycan matrix synthesis. Furthermore, as shown in immunocytochemistry assay (***Figures 6,7***) and immunoblotting assay (***Figure 8***), in the presence INS with ABT-761, 5-LO protein decreases as in the presence of inhibitor ABT-761 only (***Figure 9,10).*** The markedly negative effects on chondrogenesis of ABT-761 can contribute to the bone formation by accelerating apoptosis of cartilage, which is explained by the fact that during chondrogenesis, to promote endochondral ossification, the mature chondrocytes or cartilage cells must undergo hypertrophy, then apoptosis to allow cartilage

become vascularized and osteoblasts invade the cartilage tissue (Quintana, zur Nieden et al. 2009). From other research that shows that 5-LO causes acceleration in the process of endochondral ossification and results in the earlier detection of late chondrocyte genes (Cottrell and O'Connor 2009).

Overall, our experiment results show that treatment with 1μM ABT-761 has no effect on chondrogenesis, neither increase nor decrease, in ATDC5 cells, a mouse chondrogenic cell line. In contrast, concentrations of 10 μM and 100μM ABT-761 has negative effects on chondrogenesis through which ABT-761 promotes chondrocyte apoptosis that may potentially lead to accelerating endochondral ossification or long bone formation in patients with a long bone fracture. However, ABT-761 may not be a clinical value to regenerate or promote chondrogenesis in patients suffering from osteoarthritis.

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