The Effect of NSAIDs *in vitro*

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The Effects of NSAIDs *in vitro*

January-March 2017 Elizabeth Rohde

Abstract

The effects of non-steroidal anti-inflammatory drugs (NSAIDs) were examined *in vitro* on PLC5 human hepatocyte cells during two repeated experiments using various clinical-dose treatment concentrations of sodium salt Ibuprofen. A third experiment was carried out in which the conditions of inflammation were mimicked by adding human interferon-α in conjunction with a high clinical dose of Ibuprofen. It has been determined that long-term exposure at high physiological dose concentrations of Ibuprofen negatively affects hepatocyte viability.

Introduction

Anti-inflammatory remedies have been integral in the history of medicine and pharmacology. Natural remedies have been used for centuries, such as willow bark for relief of pain, inflammation, and fever. Plant remedies have transferred into various uses in modern medicine as active plant compounds have been identified, synthesized, and developed (Rainsford, 2007). The pioneer non-steroidal anti-inflammatory drug (NSAID) in history was Aspirin. Once salicylate was discovered as the active ingredient in willow bark and other similar plants, it was isolated, synthesized, and later commercialized by Bayer as Aspirin in the 19th century (Rainsford, 2007). With Aspirin as a main origin, other laboratory-developed NSAIDs arose in the 1950s-1960s, notably Ibuprofen. Other competing NSAIDs that arose during the same time as Ibuprofen had multiple side effects including upper gastrointestinal tract ulcers and bleeding. In the midst of this time period of innovation, Ibuprofen was the first NSAID other than Aspirin to be approved for over-the-counter sale in the early 1960s (Rainsford, 2007).

During the pre-prostaglandin era, the NSAID category of drugs was classified via results on animal models. Their chemical structure along with their therapeutic properties (anti-inflammatory, anti-pyretic, and analgesic) served as classification criteria (Rainsford, 2007). In 1991, the discovery of two classes of “cyclooxygenase” (COX) enzymes (COX-1 and COX-2) paved the way for the development of COX-2 specific NSAIDs to avoid the characteristic NSAID-related side effects (Rainsford, 2007). COX-1 is known as the constitutive isoform because it is present in most tissues and serves in promoting “good” prostaglandins for basic homeostatic functions such as gastric mucosal

integrity, regulation of renal blood flow, and more. COX-2 is known as the inducible isoform because it is undetectable in most tissues, but it can be induced rapidly by a multitude of cells involved in inflammation such as vascular endothelium, fibroblasts, and monocytes in response to cytokines, hormones, toxins, etc. COX-2 is involved in the production of "bad" prostaglandins involved in symptomatic inflammation, fever, and vasodilation (Department of Pharmaceutical Sciences, *et al*., 2003). While COX-2 specific drugs bypassed the GI ulcers and bleeding side effects that COX-nonspecific drugs initially had, they developed side effects of their own such as stroke and sudden myocardial infarction. Most COX-2 specific NSAIDs now have black box FDA warnings and there are other warnings about all NSAID-related side effects in place for over-the-counter NSAIDs (Rainsford, 2007).

Another side effect of NSAIDs is rare but sudden liver necrosis due to hepatocyte injury. Patients with chronic autoimmune diseases such as rheumatoid arthritis commonly use NSAIDs to treat their symptoms, but could be hypersensitive all NSAIDs or to a particular class of NSAIDs. Other patients could develop hepatotoxicity by taking NSAIDs in conjunction with other reactive drugs. Regardless, hepatotoxicity due to NSAID usage is a sudden and lethal complication. In O'Connor, *et al.* in 2003, researchers found various mechanisms in which NSAIDs are responsible for hepatocyte damage and death *in vitro*. At various metabolic breakdown stages, structural components of NSAIDs can cause uncoupling of oxidative phosphorylation and overall decrease of cellular ATP, mitochondrial swelling, generation of reactive oxygen species, and ultimately cytoxicity and cell death.

I work as a certified phlebotomist and laboratory assistant at Penn State St. Joseph Medical Center in Bernville, Pennsylvania. Upon encountering about fifty or more patients per day, I started to notice that an increasing amount of patients were being admitted for non-steroidal anti-inflammatory drug (NSAID) related organ damage such as stomach ulcers, kidney damage or failure, and liver problems. These issues had accumulated in the patients due to daily over the counter NSAID use such as Advil. Family members and friends of mine also consume NSAIDs daily to counteract daily aches and pains; in other cases people are actually prescribed daily NSAID use by their family doctors. Knowing the possible side effects and risk of hospitalization, I wanted to

research this area to discover how exactly a drug deemed “safe” for daily use actually interacts with cells in culture. It was decided to use the common NSAID, Ibuprofen, in three repeated experiments *in vitro* to see the direct effect of the drug on hepatocyte viability.

Methods

During the Interim 2017 ACRE, week one was spent optimizing the PLC/PRF/5 (ATCC CRL-8024) human hepatocyte line kindly shared with us by Clint Stalnecker, Ph.D. from Cornell University. Growing the cells after they were thawed proved to be difficult at first. A 1% penicillin and streptomycin mixture (Penstrep) of antibiotics were added to all media used midway through week one due to the cells having issues proliferating.

Experiment #1:

Experiment #1 was seeded during the first week of research at 0.5_E10^5 cells/mL (Figure 1).

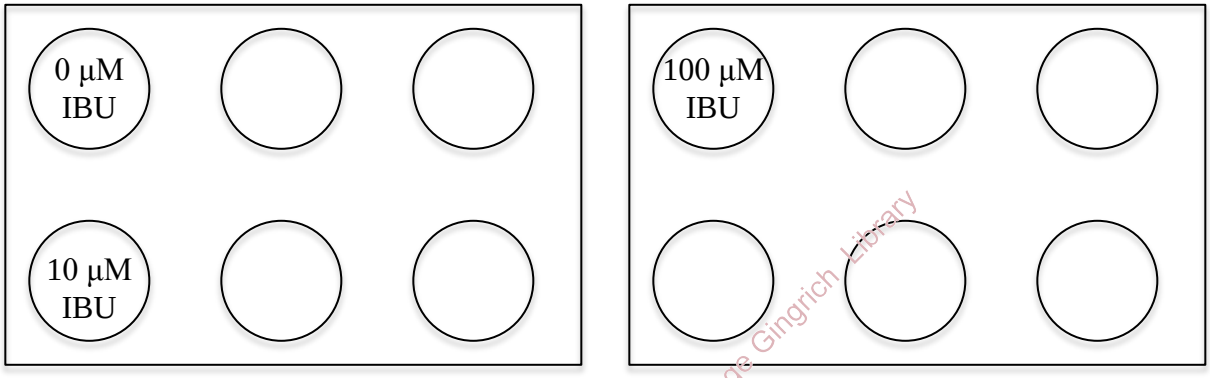

Figure 1: Experiment #1 set up in six 6-well plates. Each set of IBU concentrations was carried out in triplicate in 1%, 5%, and 10% FBS DMEM conditions.

The first run of Experiment #1 was an 18-hour 1 µL/mL IBU treatment (2 µL/well) on 6-day old cells that were subconfluent in the 6-well plates. After the 18-hour treatment, the cells were trypsinized with 0.5% trypsin for 6-7 minutes per plate and counted via hemocytometer using of trypan blue vital dye in a 1:2 dilution with the cells. Experiment #1 was then repeated with the same IBU and DMEM conditions, but as a 24-hour IBU

treatment. The PLC5 cells were seeded at 1.5_E10^5 cells/mL for the repeat experiment due to the cells of first run of Experiment #1 being subconfluent.

Experiment #2:

Experiment #2 was seeded during the third week of research at 0.5_E10^5 cells/mL (Figure 2). The low seeding density allowed the cells to proliferate as the long-term experiment developed.

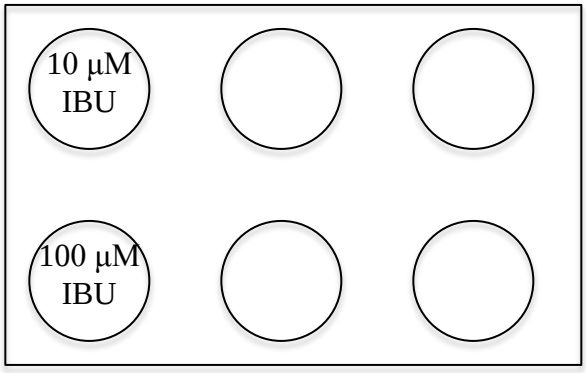

Figure 2: Experiment #2 set up in four 6-well plates. Each IBU concentration set was carried out in triplicate in 1% FBS DMEM.

Four 6-well plates were set up for a long-term experiment. Each plate was counted on a different day and the other plates were maintained. For eight days, IBU was added to fresh media at 1 μL/mL IBU treatment (2 μL/well) every 24 hours. The counts took place on days 1, 4, 6, and 8. Experiment #2 was then repeated using the same conditions and seeded at the same density.

Experiment #3:

Experiment #3 was seeded mid-March to conclude the experimental series at $1.5x10^5$ cells/mL (Figure 3). The higher seeding density allowed for faster cell proliferation in order to add the treatments to 4-day old confluent PLC5 cells in 1% FBS DMEM with added Penstrep. This experiment was also repeated at the same seeding density, but the treatments were added to 2-day old cells. The hIFN-α was added to the cells 24 hours before adding the IBU to prime the cells and mimic the conditions of inflammation. The 100 μM IBU treatment (1 μL/mL) was subsequently added to the

unchanged media with the hIFN-α for a 24-hour IBU treatment. The hIFN-α was therefore in contact with the cells for a full 48 hours.

This 24-hour IFN priming exercise was determined from Dr. Adam Hersperger's familiarity with interferon use. The concentration of interferon was determined to be slightly above the clinical ranges of Huang, et al., 2017 due to the inability to dilute the hIFN-α stock availible to us.

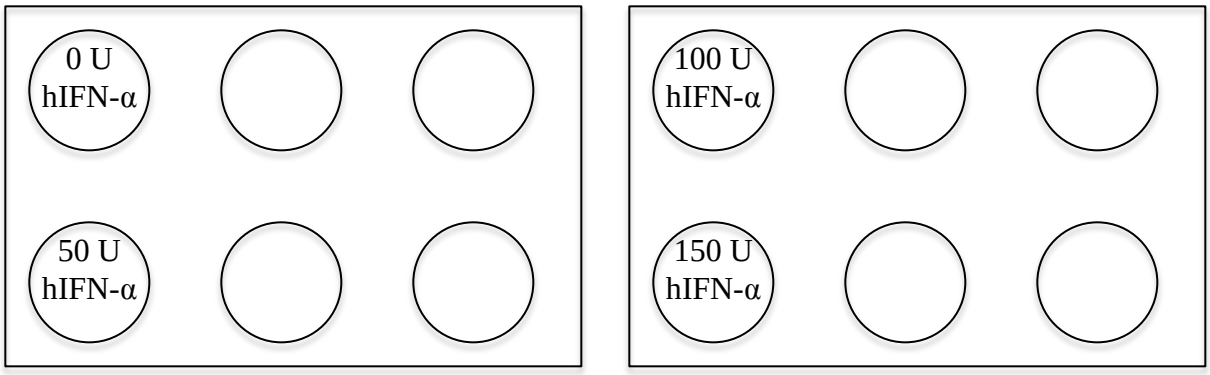

Figure 3: Experiment #3 set up in two 6-well plates. Each hIFN-α concentration was carried out in triplicate and primed for 24 hours before the addition of IBU. 100 μM IBU (2 μL/well) was then added in conjunction with the hIFN-α for a 24 hour treatment.

Results

Experiment #1:

Experiment #1 was run to determine the concentration of fetal bovine serum (FBS) in the DMEM at which the PLC5 hepatocytes were most sensitive to the drug treatments. This media FBS concentration was determined to run all future experiments with. It was hypothesized that 1% DMEM would allow the hepatocytes to be the most sensitive to the drug. This was the case in both runs of Experiment #1. The percent viability decreased proportionally to the increasing amount of IBU in the 1% FBS DMEM condition (Figures 4 and 5). The 1% FBS concentration was therefore used for the remaining experiments.

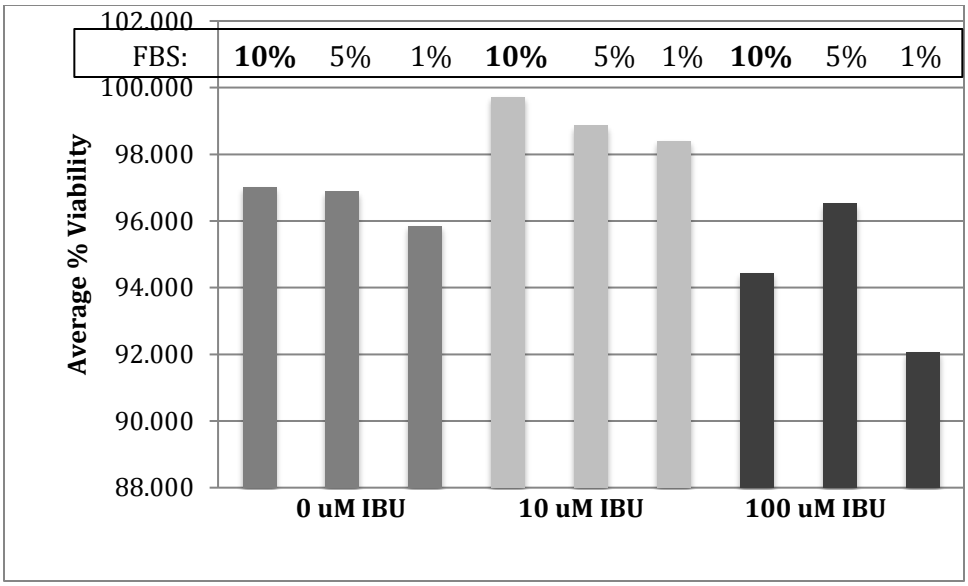

Figure 4: 18-Hour Treatment Experiment #1: The average percent viability for each well was calculated at each concentration of IBU. The 18-hour IBU treatment did show proportionality between decreasing FBS in the DMEM, but the treatment was not long enough to be definitive.

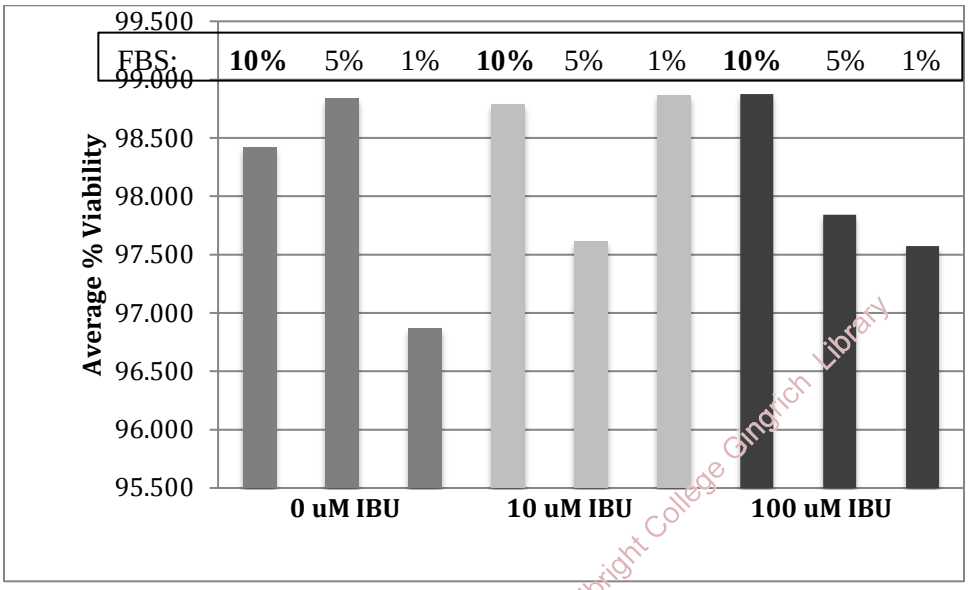

Figure 5: 24 Hour Treatment Experiment #1 Repeat: The average percent viability for each well was calculated at each concentration of IBU. The results shown here confirmed that the 1% FBS DMEM allowed the most drug sensitivity due to the proportional decreasing viability with the increasing IBU concentrations.

General observations of the cells during counting revealed that with increased IBU concentrations, the cells were generally smaller, had a greater instance of large cellular

vacuoles, and multinucleated cells were common. Blue speckles of where the trypan blue dye was adhering to spots on the cells were quite obvious.

Experiment #2:

Experiment #2 was hypothesized to show an increase in cytotoxicity and an overall decrease in viability as the duration time of IBU treatment increased. The results did show a decrease in viability over all by day 4. The higher concentration of IBU did show a much more stark decrease in viability over time (Figures 6 and 7).

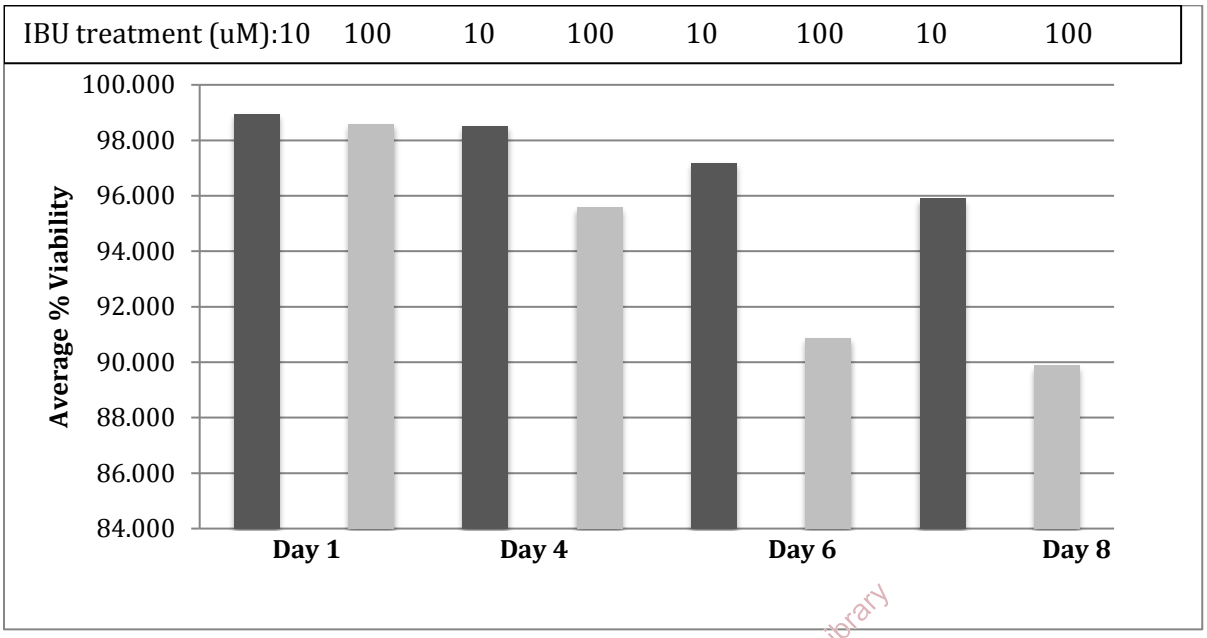

Figure 6: 8 Day Hour Treatment Experiment #2: Cellular viability decreased over time and with increasing IBU concentration in 1% FBS DMEM.

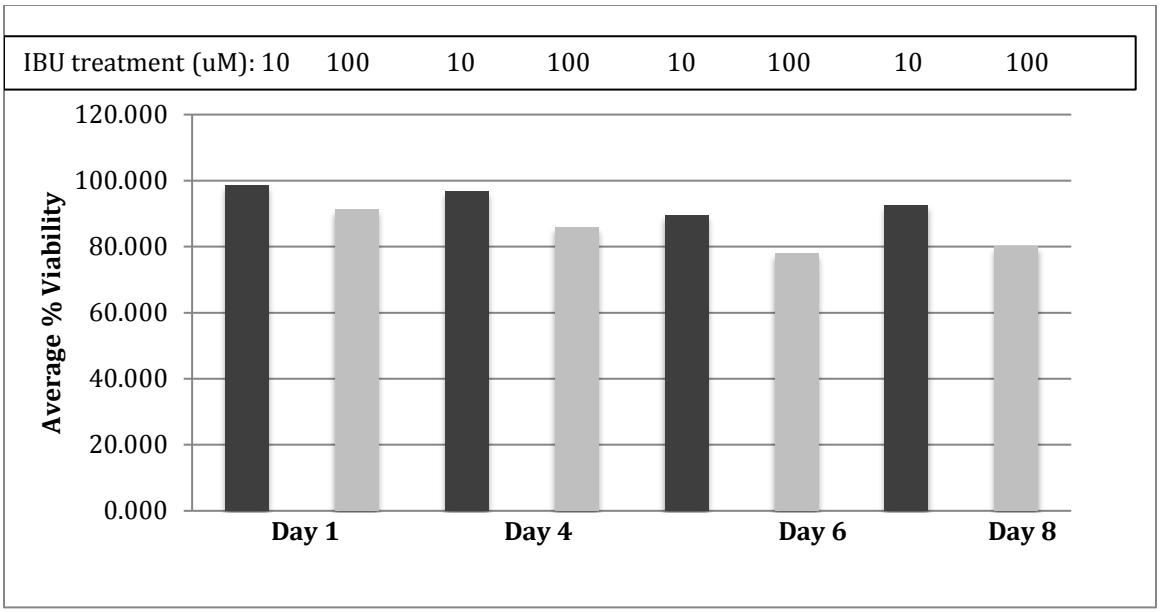

Figure 7: 8 Day Hour Treatment Experiment #2 Repeat: It was confirmed that cellular viability decreased over time and with increasing IBU concentration in 1% FBS DMEM.

General observations of the cells growing *in vitro* revealed an increasing number of cells floating in the media with increasing IBU concentration. The first count on day 1 showed similar results to the 24-hour treatment during Experiment #1. As the cells became more confluent and with increasing IBU concentrations, more cellular vacuoles and blue dye speckles on the cells were more common.

Experiment #3:

It was hypothesized that by mimicking inflammation with the addition of a known inflammatory cytokine INF-α, cytotoxicity would increase and cellular viability would decrease with increasing concentrations of hIFN-α. The first run of experiment #3 did show this trend with the exception of the highest IFN concentration (Figure 8). The second run of this experiment was very inconclusive due to the early addition of the treatments to subconfluent 2-day old cells rather than the confluent 4-day old cells.

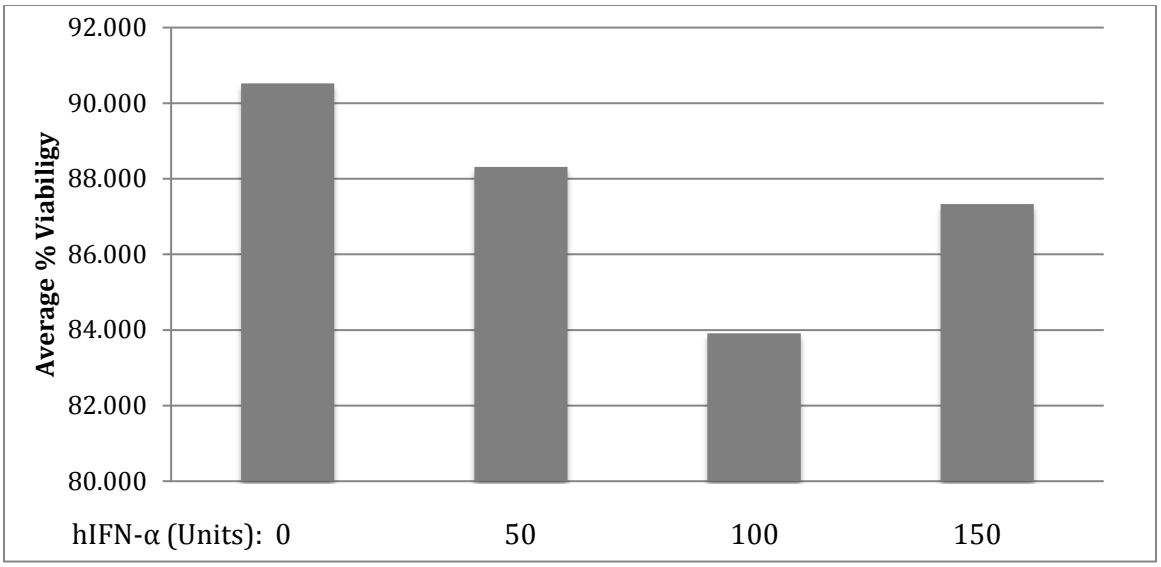

Figure 8: Experiment #3: 48 Hours of IFN- α Priming in combination with 24 Hour 100 μM IBU Treatment: Cellular viability decreased with increasing concentrations of hIFN-α with the outlier of 150 U IFN. The repeat experiment results were inconclusive.

Discussion

After consulting with Dr. Andrew Samuelsen, our goals for the research project were: to see how the NSAIDs interact with cells in culture and quantify viability using trypan blue dye exclusion assays, to specify how long it takes and at what dosage it will take (using daily cell treatment) for the cells to be damaged by sodium salt Ibuprofen (IBU) in solution, and to mimic inflammation *in vitro* with the addition of human interferon-α (hIFN-α). We had obtained the cell line and sodium salt Ibuprofen (IBU) during the end of November. We obtained the hIFN-α at the beginning of March to add to the human PLC5 hepatocyte culture in conjunction with the IBU treatment to mimic the symptoms of inflammation *in vitro*.

The PLC hepatocytes are hepatoma-derived human epithelial cells. They were slow growing in week one due to light seeding amounts; they also had a difficult time growing without antibiotic treatment. We therefore added a 1% combination of penicillin and streptomycin (Penstrep) antibiotics to the initial cultures to expand the line for optimal experimental use. Liver cells were used because they are the functional units of the major organ of the body that metabolizes drugs and detoxifies the body. There are also clinical cases of hepatocyte damage and death due to NSAIDs. The first experiment was also set up at the end of week one.

Experiment #1 was first carried out as an 18-hour IBU treatment (Figure 4), and then repeated as a 24-hour IBU treatment (Figure 5). This experiment was mainly for optimizing the percentage of growth factor Fetal Bovine Serum (FBS) used in the DMEM. The lower the percentage of FBS, the more sensitive the cells were to the Ibuprofen treatments. This sensitivity was determined by using a control and dosing at 1 μL IBU/mL medium with two concentrations of Ibuprofen (10 μM and 100 μM, respectively). The cells were then counted and assessed for viability after the treatment. These IBU concentrations were derived from the physiological concentrations of the drug in the human body during regular dosage and also what was used *in vitro* on experiments using HepaRG cells in Truisi *et al*., 2015. We therefore decided to use 1% FBS DMEM for further experiments comparing drug concentrations based on the results obtained in Experiment #1 and Experiment #1 repeat.

Experiment #2 was an 8-day continuous Ibuprofen treatment (Figure 6) at the 10 μM and 100 μM IBU concentrations in 1% FBS DMEM with 1% penstrep. The10 μM concentration acted as the control because no adverse cell death should happen at such low levels of IBU, which was confirmed in Experiment #1, and 100 μM IBU was determined to be the damaging IBU treatment concentration from Experiment #1. The medium was changed and new IBU was added daily for the 8-day time period as the cells metabolized the drug. The cells were counted on days 1, 4, 6, and 8 to get a conclusive data set. Cell counting was carried out by using a vital stain, trypan blue, to assess cellular viability (as % Viability). The dead cells take up the blue dye as it penetrates the cell membrane and the live cells exclude the dye. The results so far have concluded that as Ibuprofen concentration increases over time, the cellular percent viability decreases. Experiment #2 was repeated and showed very similar results to the first run (Figure 7). The observations of increased number of floating cells in the media as confluency and IBU concentration increased showed that the hepatocytes were dying and dis-connecting from the bottom of the culture. Due to the media changes and trypsinization processes, the floating cells were not accounted for in the viability counts. It was also speculated that the blue speckles on the cells at increasing IBU concentrations were spots of necrotic cell membrane that absorbed the trypan blue dye.

Experiment #3 was done after the interim period in mid-March as an independent study. Three increasing concentrations of human interferon-α were added to the PLC5 cells for a 24-hour priming period, then without a media change, 1μL/mL of 100 μM IBU was added for a 24-hour treatment. The addition of hIFN-α, a cytokine involved in the inflammatory process, was to mimic the conditions of inflammation *in vitro* before and during the IBU treatment. This is because humans consume NSAIDs to treat inflammation, so it would be a natural state in which drug use would occur. The concentrations of hIFN-α were slightly elevated from the measured clinical serum concentrations in Huang, *et al*., 2017 due to the inability to dilute the interferon stock to such small quantities. It was also hypothesized that interferon and general cytokine concentration would be greater at the target organ of action than in blood serum. The first run of Experiment #3 did show a trend in which average cellular viability decreased with increasing interferon concentrations. There was an outlier at the 150 U interferon concentration (Figure 8). Experiment #3 was repeated to confirm the results, but the repeat was deemed inconclusive due to the treatments being added to subconfluent cells. They did not behave and proliferate in the same manner with the addition of interferon.

In the future, I would like to run Experiment #2 with a 0 μM IBU control to confirm that low doses of IBU do not damage hepatocytes. I would also like to repeat Experiment #3 on confluent cells to confirm the results found. To expand on this research in the future, the effects on cellular viability of IBU should be compared to the effects of other NSAIDs; this could include the same NSAID class and also different NSAID classes. The use of another inflammatory cytokine in conjunction with hIFN-α or alone with NSAIDs would also be interesting to see. Also, in today's age of innovation, it would be very interesting to test out different drug classes other than NSAIDs to compare cytotoxicity such as the well-known acetaminophen. Newer drugs or other plant-based drugs such as circumin could also be tested. Finally, the reason of cellular death could be possibly measured via flow cytometry. All of these future applications could be used to re-evaluate the safety of over-the-counter analgesics as well as to possibly validate overlooked natural remedies that have less overall side effects than NSAIDs.

In conclusion, PLC5 hepatocyte viability decreased over time with increasing concentrations of Ibuprofen. Cellular viability also decreased with increasing

concentrations of human interferon-α, though the results are inconclusive. Overall, regular use of NSAIDs poses many risks, side effects, organ damage, and rarely death due to these complications. This class of over-the-counter drug should be re-evaluated for safe dosage and use as NSAIDs become more popularly used in modern culture.

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