IDENTIFYING PROMOTERS OF HEPATIC REGENERATION IN ZEBRAFISH (DANIO RERIO) FOLLOWING ACETMINOPHEN LIVER ABLATION

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[To the students of the Georgia Institute of Technology]

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LIST OF SYMBOLS AND ABBREVIATIONS

APAP	Acetaminophen
DPF	Days Post Fertilization
GFP	Green Fluorescent Protein
PTU	1-phenyl 2-thiourea
ALF	Acute Liver Failure
NAC	N-Acetylcysteine
NAPQI	N-acetyl p-benzoquinone

The names of the compounds used and found promising as well as the cellular pathways they affect are being withheld at the request of my lab.

CHAPTER 1

INTRODUCTION

Identifying chemicals that can promote the regeneration of liver tissue would be important for curing various liver diseases and ameliorating liver damage in humans. Due to their transparency, *Danio rerio* (zebrafish) embryos are a useful tool for studying organ regeneration. In order to study the regeneration of the liver using zebrafish, a method of destroying or ablating the embryo livers is necessary. Currently the methods for destroying zebrafish livers to observe the regeneration fall under two categories, hepatectomy (surgical removal of liver tissue) or organ specific chemical ablation. Previous methods of specific organ ablation in zebrafish models use transgenic lines that are made to express a certain enzyme that can then convert a normally non-toxic chemical into a toxic form [1]. This method is difficult because of the requirement of special transgenic lines.

Acetaminophen (APAP) is one of the most common over-the-counter pain medications in the world [2]. Although it is normally used to treat pain, overdoses have been found to cause hepatotoxicity and acute liver failure [3]. Based on this information, acetaminophen administered in water was explored as a possible easier method of directed liver ablation in zebrafish embryos. Because acetaminophen damage causes acute liver failure, zebrafish liver ablation using this drug could be a model for studying acute liver failure, and testing possible antidotes/drugs to cure the disorder.

A transgenic zebrafish line expressing a green fluorescent protein (GFP) in the liver was used to assess the damage and regeneration of the livers of tested embryos. Treating zebrafish embryos from 2-4dpf (days post fertilization) with various concentrations of acetaminophen, it was found that acetaminophen at certain concentrations can be used effectively to ablate embryo livers without killing them.

This model was then used to destroy the livers of embryos used in a large-scale chemical screening of possible regeneration promoting chemicals. Compounds were screened first from a Tim Tec compound library. Because the cellular targets of each of the novel compounds in the Tim Tec library are not known, further screening using a

compound library targeting stem cell signaling pathways was performed in order to have a more controlled and directed screening. The compounds from both libraries were tested at 50µM, but due to high mortality at this concentration, further screening of the Stem Cell plate was performed at 25µM in an attempt to reduce the toxicity of the compounds. Through treatment of the injured embryos from 4-5dpf, several candidates of liver regeneration promoters were found. The chemicals from the stem cell library that were deemed promising were tested again at 50µM, 25µM, and 12.5µM to confirm their efficacy. The wider range of concentrations revealed several chemicals as possible promoters of hepatic regeneration. A trend was observed that many of the promising compounds affect the same signaling pathway in the stem cells. This finding revealed some pathways that are promising to have strong effects on the proliferation and differentiation of hepatic stem cells following a chemical injury. If further testing confirms the efficacy of the promising compounds, their ability to promote regeneration of hepatocytes could eventually lead to these compounds being formulated into drugs or treatments for humans.

CHAPTER 2

LITERATURE REVIEW

The regenerative property of the human liver has been common knowledge for thousands of years. The concept of liver regeneration can be traced as far back as ancient Greek mythology. In the legend of Prometheus, after defying the gods, the Titan Prometheus was sentenced to be bound to a rock where an eagle would eat his liver, which would grow back every morning, and create an eternal cycle of punishment.

In reality, vertebrate livers do not regenerate based on the technical standards of regeneration. True regeneration occurs through the development of a blastema at the injury site that is made up of undifferentiated cells. The cells divide and differentiate and eventually the form and function of the tissue is restored [4]. Vertebrate livers regrow through a process called compensatory growth. During this process, cells in the liver divide in order to replace the mass that was lost, however, while the general size and mass of the liver may be restored to normal, the new growth does not reform the lost structures and lobes exactly [4].

Livers are comprised mainly of parenchymal cells known as hepatocytes. The hepatocytes are the cells that are responsible for the main functions of the liver including protein storage, detoxification of substances, transformation of carbohydrates, and the synthesis of proteins, bile salts, cholesterol, and phospholipids. When a mass of hepatocytes is removed through a hepatectomy, the remaining cells begin to divide to replenish the lost mass. When hepatocytes are destroyed due to chemicals or toxins, the tissue is replaced through the replication and differentiation of hepatic progenitor/stem cells[4].

Acute liver failure (ALF) is a relatively rare condition that is characterized by the rapid loss of hepatic function. Excessive alcohol consumption, drug/toxin induced liver damage, various metabolic problems, and many different viral liver diseases, such as hepatitis, can cause ALF. ALF can lead to cerebral edema, bleeding disorders, and hepatic encephalopathy. These complications can quickly lead to coma and death in patients.

Currently, the only method of effective therapy for ALF is liver transplantation. According the U.S. ALF study group, from 1998-2007 44% of patients with the disease were placed on a transplant list [5]. Of the 44% placed on the list, 25% received a transplant and had a survival rate of 70% over the next year. Of the remaining patients, 10% died on the waiting list, and 9% recovered spontaneously. In total 30% of the patients in the study group died of the disease. In 2011, the waitlist to receive a liver transplantation was over 16,000 patients long in the United States alone. During this same year, only 39% of patients on the transplant waitlist received a transplant, and 15% of patients died waiting [6]. Although the survival rate of those who received the surgery was very high (89.5%), the small percentage of patients who received treatment makes transplantation ineffective as the primary method of treating the patients in need. This represents a need for a more simple and effective method of treating acute liver failure.

Acetaminophen is one of the most commonly used analgesics in the over-the-counter drug market today. It is the main ingredient in common brand name pain medicines like Tylenol. Acetaminophen overdoses were the cause of 46% of the ALF cases between 1998 and 2007 among adults in the US [5]. Overdoses of acetaminophen alone or in combination with other drugs cause over 300 deaths annually [7]. Acetaminophen is not only the most common drug induced cause of ALF, but also the most common cause in general.

Ingested acetaminophen is converted into a toxic metabolite, N-acetyl p-benzoquinone (NAPQI), by cytochrome p450 enzymes in the liver [3]. At recommended doses, NAPQI is inactivated by glutathione, but during an overdose, unconjugated NAPQI leads to dysfunction of critical liver cell proteins, oxidative stress, and mitochondrial damage. These damages lead to necrosis of hepatocytes, which causes acute liver failure.

Current research shows that other than transplantation, the only somewhat effective therapy for treating acetaminophen caused liver damage is N-Acetylcysteine (NAC). NAC is normally administered to patients who seek help quickly after an acetaminophen overdose. NAC has been shown to reduce mortality in acetaminophen overdose situations by 20-28%, but only has a window of efficacy within 12 hours of the ingestion of the acetaminophen [3]. Treatment of NAC after the effective window or

prolonged treatment has been shown to have a negative influence on the outcome and survivability of the patient. The restricted time in which NAC can be used as an effective antidote for acetaminophen liver damage shows a necessity for the exploration of other chemicals or compounds, which might induce proliferation and regeneration after injury.

The damage of acetaminophen on hepatocytes is chemical, and therefore the regeneration of the liver occurs through the replication and differentiation of hepatic progenitor/stem cells. By performing a wide chemical screening of novel compounds, especially ones known to target various cell-signaling pathways in liver stem cells, this project could identify potential compounds that can be used to promote the regeneration of the liver after a chemical injury. Discovering promising novel compounds during this screening could also help identify which cell signaling pathways may be critical in promoting the growth, replication, and differentiation of hepatic stem/progenitor cells to create new tissue. Further studies confirming the efficacy of any promising compounds could lead to the development of more successful and consistent methods of treating patients suffering from acute liver failure, or acetaminophen overdose, without the constraints, dangers, and invasiveness of transplants.

CHAPTER 3

MATERIALS AND METHODS

Materials

The fish embryos were obtained from an in-cross of transgenic zebrafish line "GFP". This transgenic line was helpful to the experiment because these fish express a green fluorescent protein in the cytosol of their hepatocytes. This fluorescence allows for the visualization liver size in the developing embryos. In order to keep the embryos transparent for the period of the experiment, their water was treated with 1M 1-phenyl 2-thiourea (PTU). PTU prevents melanogenesis in the embryos by blocking all of the tyrosine-dependent steps in the pathway [8]. Acetaminophen was used to ablate the livers of the test embryos. Chemical screening was performed using novel compounds from three plates from Tim Tec library (88 compounds each for 264 total), as well as a 75 compound plate of stem cell targeting novel compounds.

Obtaining Embryos

Several mating tanks of GFP fish were assembled. These tanks contained one male and one female fish and were left overnight for fertilization to occur. The next day (Day 0) the embryos were harvested into petri dishes with about 80 embryos per dish. The egg water was replaced with 20ml of new egg water and the embryos were placed in a 24^{o} incubator overnight. On Day 1, the egg water was removed and replaced with 19ml of egg water and 1ml of 1xPTU solution per plate in order to prevent pigmentation.

Acetaminophen Dose Experiment

On Day 2, the dishes were split into four groups in a six well plate, one control and three experimental. Each group contained 25 embryos. The control embryos were left in only egg water. The other three had their egg water treated with acetaminophen to make 5µM, 10µM, or 25µM solutions. The embryos were left in the incubator until Day 4. On Day 4, the embryos were removed and split up into a glass 9 well plate. They then were observed using a fluorescent microscope, and images of the fluorescent livers were recorded for each test group. All pictures were taken from the ventral side of the fish, at

the same magnification (100x). The comparative size of the livers in each group was estimated from the 2D image of the ventral side of the fish.

Chemical Screening

Tim Tec-

The embryos underwent the same procedure as above for the 10mM acetaminophen treatment up to day 4. On day 4, the test embryos were washed to stop ablation and placed in clean egg water. Images were taken of the control and ablated embryos for the pre-treatment size of the livers. Embryos were then placed in a 96 well plate with 3-5 embryos in each plate along with 199µl of egg water. Wells in column 1 are controls with 1-A through 1-D being unablated controls and 1-E through 1-H being ablated embryos that will not be treated with chemicals. The embryos in columns 2-12 then had 50µM (1µl diluted in 199µl egg water) of the chemical in the corresponding well of the novel compound 96 well plates added to them. The 96 well plates were then wrapped in foil to block out the sun and placed in an incubator overnight. On day 5, images of the livers of all of the surviving embryos were obtained. Images were not recorded for wells in which the embryos died or did not show visibly larger livers than the acetaminophen control wells.

Stem Cell-

The same general procedure was followed as performed in the Tim Tec screening except 4-8 embryos were placed in wells of 24 well plates along with 400µl egg water until enough were filled to accommodate all 75 compounds, unablated controls, and ablated controls. Originally, the stem cell compounds were screened at 50µM, but after observing high mortality among the embryos, a second trial using 25µM was performed. The promising compounds from these screening were tested a second time at using 10 embryos tested for each promising compound at 50µM, 25µM, and 12.5µM. Images were recorded in the same fashion as the ablation dose experiment and the Tim Tec screening.

CHAPTER 4

RESULTS

Acetaminophen Dosage

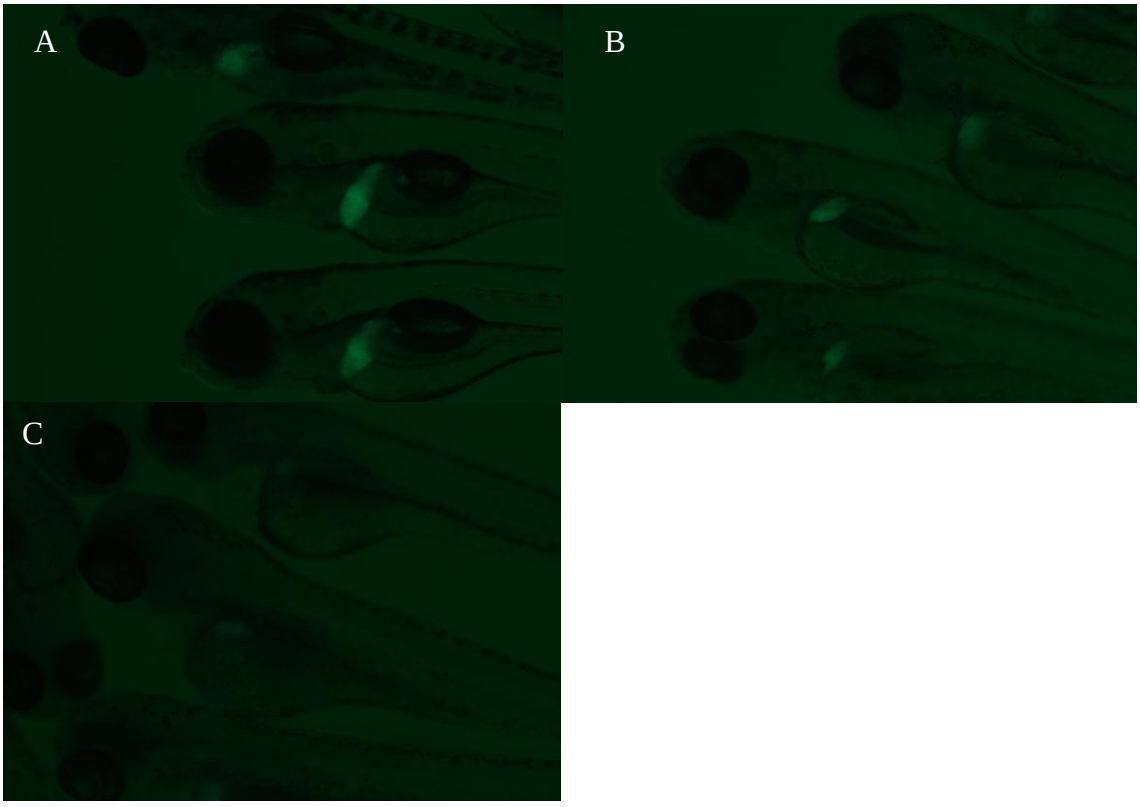


Figure 1. Acetaminophen Dose Experiment A) Control at 4dpf, B) 5mM test at 4dpf, C) 10mM test at 4dpf

Summary: As shown in Figure 1.A the control fish developed large sized livers. Figure 1.B shows that the fish treated with 5mM acetaminophen exhibited a notable decrease in liver size. The zebrafish exposed to 10mM acetaminophen showed a much more significant decrease in the size of their livers (Figure 1.C). At day 4 the survival rate of the embryos in each group was 25/25 control, 25/25 5mM APAP, 21/25 10mM APAP, and 0/25 25mM APAP.

Controls:

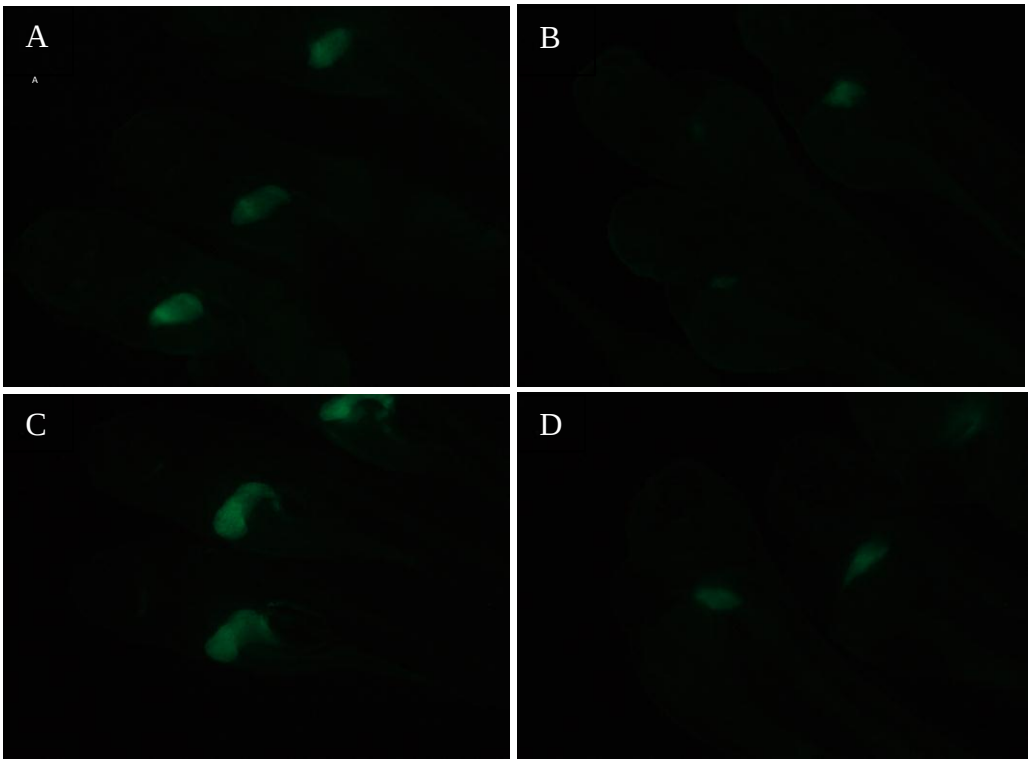


Figure 2. A) Control embryos at 4dpf B) APAP treated control embryos at 4dpf. C) Control embryos at 5dpf. D) APAP treated control embryos at 5dpf.

Plate 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	N	N	X	X	X	X	X	X	X	X	X
B	N	X	X	X	X	X	X	X	X	X	X	X
C	N	X	X	X	X	X	X	X	X	X	X	X
D	N	X	X	X	N	X	X	X	X	X	X	X
E	X	X	X	P	X	X	X	X	X	X	X	X
F	X	X	X	X	X	X	X	X	X	X	X	X
G	X	X	X	X	P	X	X	X	X	X	X	X
H	X	X	X	X	N	X	X	X	X	X	N	X

Table 1) Chemical Plate 1. X=Died, N=No regeneration, P=Promising

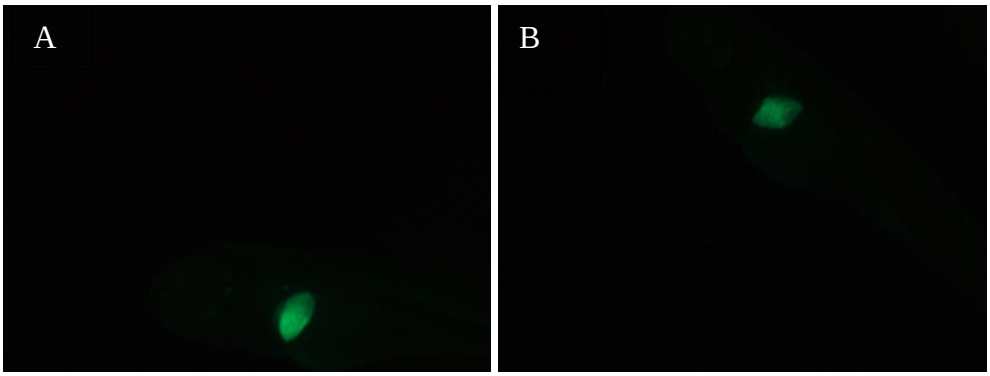


Figure 3. Tim Tec Plate 1A) Well 4E at 5dpf B) Well 5G at 5dpf

Plate 2:

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	N	X	X	X	X	X	X	N	X
B	X	X	X	X	X	X	X	X	X	X	X	X
C	X	X	X	X	X	X	X	X	X	X	X	X
D	X	X	X	X	X	X	X	X	X	P	X	X
E	N	X	X	X	X	X	X	X	X	X	N	X
F	X	X	X	X	X	X	X	X	X	X	X	X
G	N	X	X	X	X	X	X	X	P	X	X	X
H	X	X	X	X	X	X	X	X	P	X	X	X

Table 2) Chemical Plate 2. X=Died, N=No regeneration, P=Promising

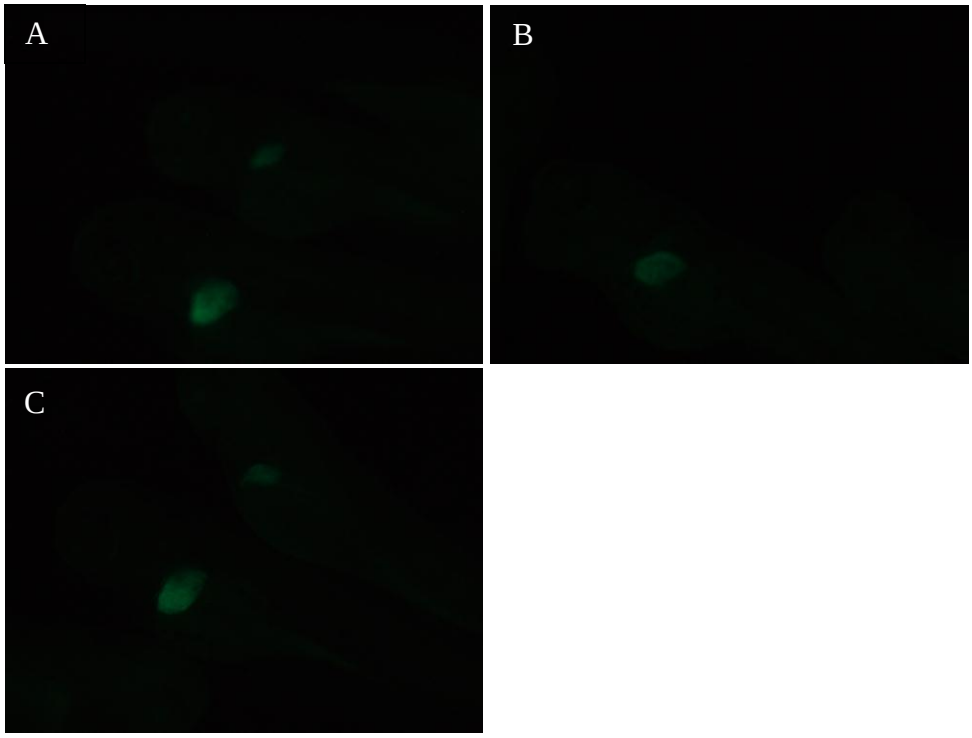


Figure 4. Tim Tec Plate 2 A) Well 9G at 5dpf, B)Well 9H at 5dpf, C) Well 10D at 5dpf.

Plate 3:

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	X	N	X	X	X	X	X	X	X	X	X
B	N	N	N	X	X	X	X	X	X	X	N	X
C	N	X	X	X	X	P	X	X	X	X	X	X
D	N	X	N	X	X	X	X	X	X	X	X	X
E	X	X	X	X	X	X	X	X	X	X	N	X
F	N	X	X	X	X	X	X	X	X	X	X	X
G	X	X	X	X	P	X	X	X	X	X	X	X
H	N	X	X	X	X	X	N	X	N	X	X	X

Table 3) Chemical Plate 3. X=Died, N=No regeneration, P=Promising

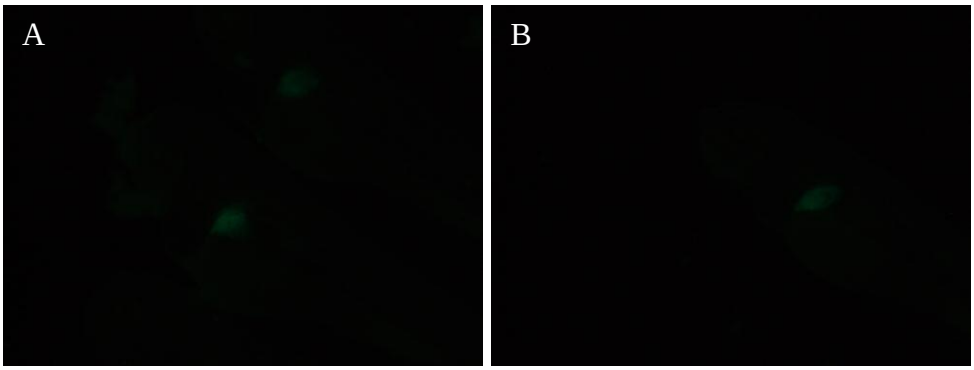


Figure 5. Tim Tec Plate 3 A) Well 5G at 5dpf, B) Well 6C at 5dpf

Summary: Three plates of novel compounds from the Tim Tec library have been screened. As shown in Figure 2, the embryos that were treated with 10mM APAP from 2-4dpf showed significantly smaller livers on day 4 and 5 compared to the controls. The remaining figures (3-5) show that, of the chemicals screened (264), there are seven that elicited significant regeneration compared to the APAP treated controls. As seen in Table 1, 2, and 3 the majority of the chemicals tested in each plate caused the embryos to die. Many of the embryos that survived the chemical treatment did not show significantly larger livers than the ablated control embryos.

Stem Cell Chemical Screening

Day 5, 50µM Chemical Trial:

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	P	X	X	X	X	X	X	X	X	E
B	P	X	X	X	X	X	X	X	X	X	P	E
C	X	X	X	P	X	X	X	X	X	X	X	E
D	X	X	P	X	X	N	X	X	X	P	X	E
E	X	P	X	X	X	X	X	X	X	X	X	E
F	N	X	X	X	X	X	X	X	X	N	N	E
G	X	X	X	X	X	X	X	X	X	E	E	E
H	E	E	E	E	E	E	E	E	E	E	E	E

Table 4) Stem Cell 50µM. X=Died, N=No regeneration, P=Promising, E=Empty Layout based on Stem Cell compound plate

Summary: Table 4 shows that the initial 50µM screening of the stem cell library revealed a few promising chemicals. The vast majority of the chemicals killed all tested embryos.

Day 5, 25µM Chemical Trial:

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	N	N	X	P	N	N	X	X	X	X	E
B	N	X	P	X	X	N	X	X	N	X	X	E
C	N	X	N	P	X	X	X	N	N	X	N	E
D	X	N	N	X	N	X	N	X	P	N	X	E
E	X	X	N	X	X	X	N	X	X	X	N	E
F	X	X	N	P	X	X	X	X	X	X	P	E
G	N	X	X	X	X	X	N	X	X	E	E	E
H	E	E	E	E	E	E	E	E	E	E	E	E

Table 5) Stem Cell 25µM. X=Died, N=No regeneration, P=Promising, E=Empty Layout based on Stem Cell compound plate

Summary: Table 5 shows that at 25µM there are still some compounds that exhibited higher regeneration of the liver compared to the control, and that fewer chemicals killed the embryos at the lower concentration.

Promising Compound Rescreen:

Mortality

Compound	50µM	25µM	12.5µM	Target
1-B	1	5	2	"A"
2-E	0	1	X	"B"
3-A	8	X	X	"B"
3-B	1	1	0	"C"
3-D	0	2	0	"D"
4-C	0	0	1	"D"
4-E	0	1	2	"B"
4-F	1	1	5	"E"
5-A	5	0	2	"C"
9-D	0	2	0	"D"
10-D	0	0	X	"F"
11-B	0	0	X	"F"
11-F	10	10	10	"G"

Table 6) Dead embryos from each well of the rescreen (10 embryos per well) and the cell-signaling pathway targeted by the compounds tested. X represents concentrations that were not screened for because the supply of the compound ran out.

Promising Regeneration

Compound	50µM	25µM	12.5µM	Target
1-B	0	0	3	"A"
2-E	3	0	X	"B"
3-A	0	X	X	"B"
3-B	3	2	2	"C"
3-D	1	1	2	"D"
4-C	1	2	2	"D"
4-E	1	2	0	"B"
4-F	0	1	0	"E"
5-A	3	2	0	"C"
9-D	0	0	2	"D"
10-D	6	4	X	"F"
11-B	3	0	X	"F"
11-F	X	X	X	"G"

Table 7) The number of embryos in each treatment well that exhibited larger livers than the day 5 ablated control. X marks treatments that were not performed or ones in which all embryos died.

Controls:

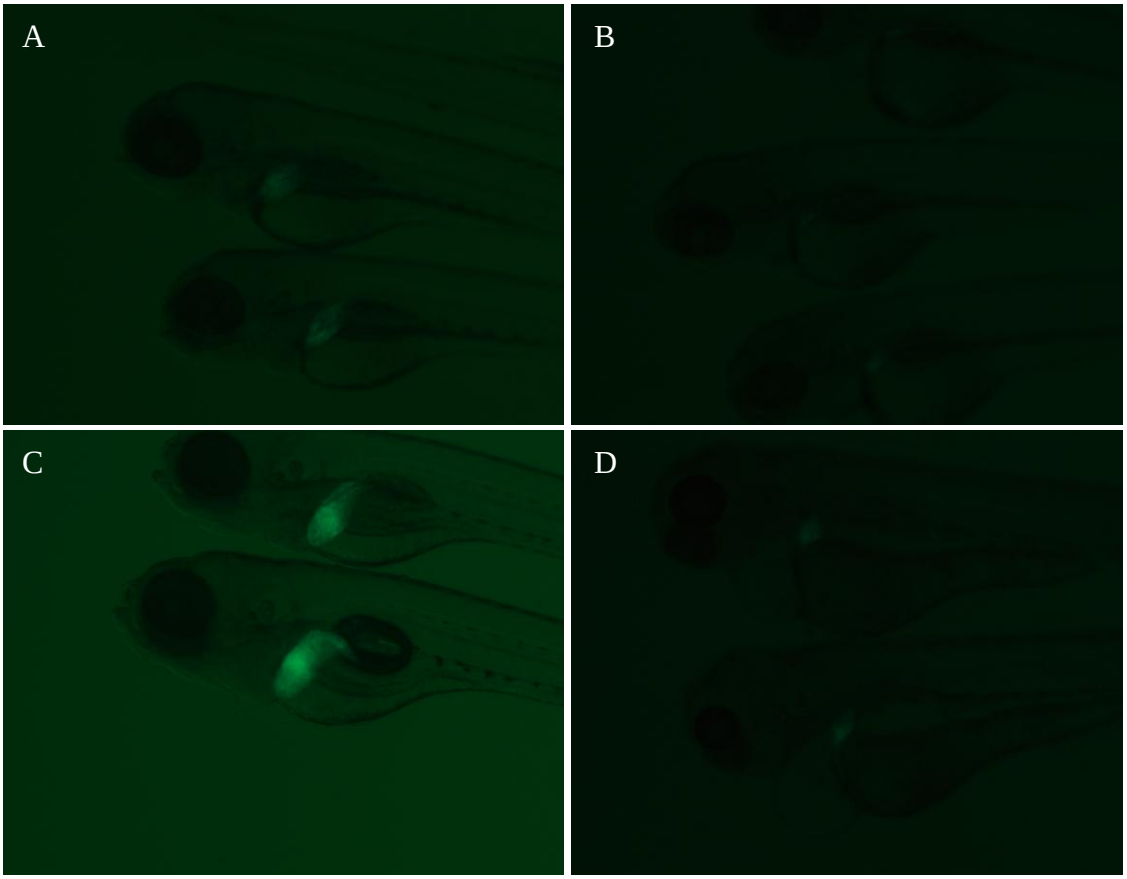
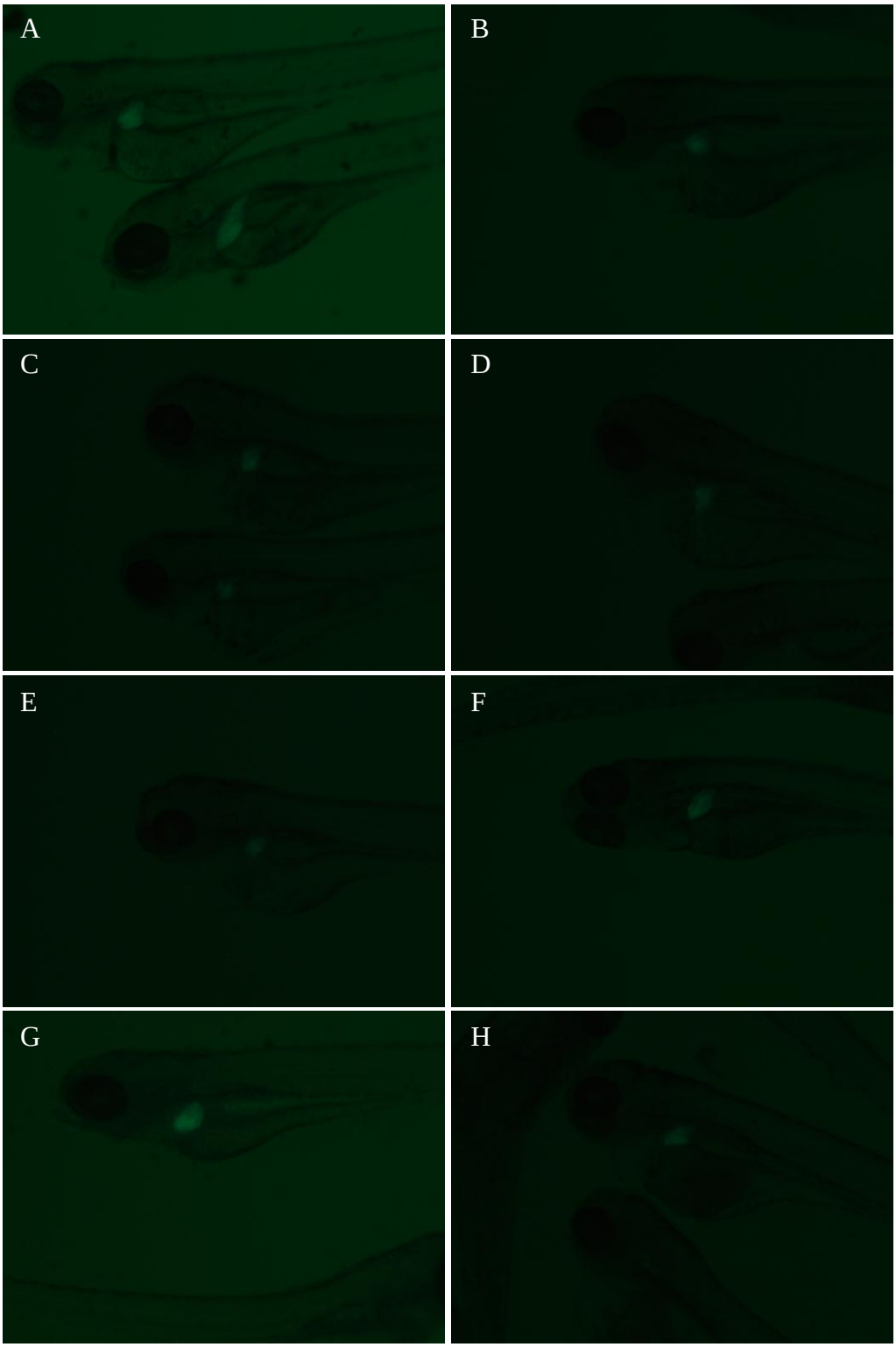


Figure 6. Rescreen Controls A) Control embryos at 4dpf B) APAP treated control embryos at 4dpf. C) Control embryos at 5dpf. D) APAP treated control embryos at 5dpf.

Compounds:



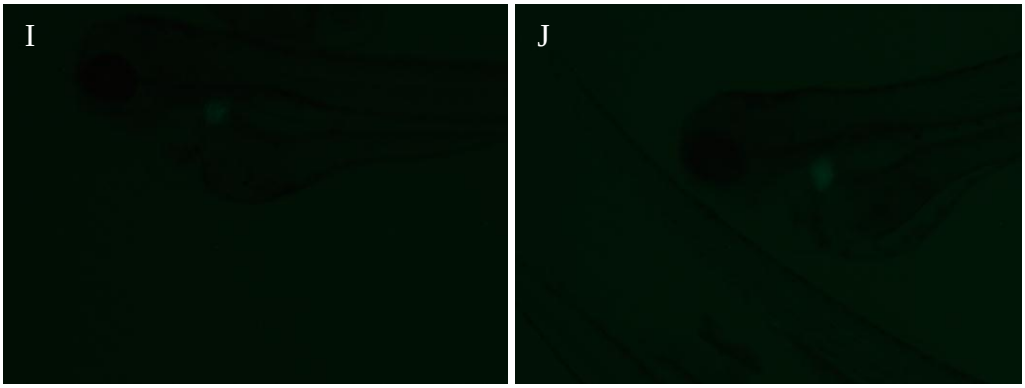


Figure 7. Rescreen Promising compounds A) 5-A 50µM, B) 5-A 12.5µM, C) 2-E 50µM, D) 4-C 50µM, E) 4-C 25µM, F) 4-C 12.5µM, G) 5-A 50µM, H) 5-A 25µM, I) 10-D 50µM, J) 10-D 25µM

Summary:

As seen in Table 6, for most of the compounds, mortality did not seem to correlate with the concentration of the compound. Some of the compounds (3-A and 11-F) previously labelled promising killed all or almost all of the embryos in the rescreen. Table 7 shows that the some of the rescreened compounds did not cause very significant regrowth in most of their embryos and may not be very effective. Compounds 3-B, 3-C, 4-C, 4-E, 5-A, and 10-D seem to be able to promote regrowth at a wide range of concentrations. 2-E and 11-B seem to be most effective at high concentrations while, 1-B appears to be most effective at low concentrations. Figure 7 shows the regeneration seen at different concentrations of one of the compounds that affected each of the five signaling pathways, which had compounds show promising regeneration. Cell pathways "A", "B", "C", "D", and "F" were affected by one or more of the compounds that caused increased regeneration during the rescreening.

CHAPTER 5

DISCUSSION

Acetaminophen Dose

As seen from Figure 1, acetaminophen administered in the water of the zebrafish embryos is effective at destroying or retarding the growth of developing livers. The concentration of acetaminophen is critical for the directed ablation. At 5mM, the drug elicited a small decrease in the liver size of the developing embryos; however, at a concentration of 10mM the liver was drastically smaller. This experiment shows that with the correct concentration, acetaminophen can be used as a method of ablating livers.

The 25mM test proves that the dose does need to be specific. All of the embryos in this test were killed, which leads to the conclusion that either the concentration of acetaminophen that they were exposed to completely destroyed the liver, which would cause death, or that acetaminophen in high concentrations can have other adverse effects on the organs of the zebrafish that could cause death. There were a few embryos that were killed by the 10mM acetaminophen, but the 84% survival rate was enough to consider 10mM the ideal concentration for the ablation model. Essentially, the theory that acetaminophen's hepatotoxicity in humans can be also expressed in zebrafish and used as a method of ablation in the liver of embryos is supported by this experiment. This method of liver ablation can be used as a simple and reasonable method to study the regeneration of livers in developing embryos.

Chemical Screening

Tim Tec-

Using the acetaminophen ablation model, 264 novel Tim Tec compounds were screened for their ability to promote regeneration of the zebrafish embryo livers from 4-5dpf. Of the 264 compounds tested, around 97% caused mortality or reduced the liver to an unobservable size. This extremely high mortality rate could have been the product of

chance and not solely be caused by the chemicals. Further screening of the same plates may be necessary to be sure that mortality was not a coincidence. The high mortality may have also been caused by the liver damage in individual embryos being too severe, and possibly beyond repair.

Seven compounds were found that caused a visible significant regeneration of the liver tissue compared to ablated embryos that were not treated with any novel compounds. These results make those seven compounds good candidates for further, more specific, tests to confirm their ability to promote liver regeneration. The cellular target of the compounds in the Tim Tec library are unknown, so many further tests will be needed to perfect their application. If further testing proves any of the seven compounds to be successful at promoting liver regeneration, the compound will have the potential to one day become a drug that could be used to treat humans.

Stem Cell-

The screening of the stem cell library revealed a few compounds that may be promising promoters of liver regeneration. The compounds shown in Figure 7 along with compounds 3-B, 3-C, and 11-C showed a noticeable level of increased regeneration compared to the ablated control embryos. The promising compounds that effected cell pathways "C" and "D" were observed to be effective at 50µM, 25µM, and 12.5µM during the rescreening. Compounds effecting pathways "A", "B", and "F" were also seen to be promising, but only at certain concentrations. This leads to the assumption that some of the pathways affecting the promotion of proliferation and differentiation of hepatic stem cells are more sensitive to changes in the concentration of one of the factors involved. Some compounds were more effective at only high concentrations, some at only low concentrations, and some were lethal at high concentrations, which could mean that the concentration of the compound for screening is very critical to the promotion of regeneration. Many of the results of the rescreen were inconsistent with the two rounds of screening at only 50µM or 25µM. Although some of the compounds seem to be promising promoters of hepatic regeneration, because of all of the inconsistencies, further screening with more repetitions and concentrations is necessary.

Conclusion

The toxicity of acetaminophen seen in humans is relatable to a zebrafish animal model. Acetaminophen can be used at specific concentrations for simple, controlled, and safe ablation of the liver of developing zebrafish embryos. The chemical screenings have identified some promising compounds to promote the regeneration of livers after an acetaminophen injury, but there is not enough consistency and repetitiveness to make concrete conclusions about any of them. Further screening of the promising compounds is necessary to confirm their effectiveness and potentially identify those that can be used to treat acute liver failure in humans.

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