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EXPLORING THE ROLE OF LIPID METABOLISM IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC) is an aggressive cancer with poor survival rates and limited treatment options whose prevalence is expected to increase up to 137% by 2030. Existing therapies outside of liver resection or transplantation have poor efficacy, and few new treatment options have been developed in recent decades. It is known that the lipid metabolism of HCC cells is significantly altered relative to normal liver cells, often involving upregulation of fatty acid synthesis and oxidation enzymes. Targeting these lipid metabolic enzymes could represent a class of novel therapeutic methods for the treatment of HCC. To investigate this possibility, we assembled a targeted library of lipid metabolic drugs and clinical compounds for a repurposing screen. We treated two liver cancer cell lines with 187 compounds targeting a diverse range of lipid metabolic enzymes in order to determine their relative anti-proliferative effects and possible therapeutic potential. Putative hit compounds were further investigated by dose response analyses and metabolic profiling by mass spectrometry. The most efficacious compounds were those identified to be targeting enzymes involved in lipid-based signaling molecule synthesis, suggesting that increased cell signaling may be a mechanism supporting enhanced proliferation downstream of lipid metabolic enzyme upregulation in HCC.

INTRODUCTION

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common form of cancer worldwide. It often not diagnosed until late stages, and therefore is difficult to treat, having a 3-year survival rate of only 19% (Greten et al., 2005). The prevalence of HCC is expected to increase 137% in developed countries by the year 2030, primarily due to changes in diet and lifestyle of the population (Estes et al., 2018). Greater rates of obesity, metabolic disorders, alcohol use, and hepatitis infection all contribute to the rising prevalence of HCC, despite new curative hepatitis therapies. Regardless of this impending surge in HCC cases, treatment options are limited. The best treatment option currently available for HCC is liver transplantation, having a 5-year survival rate of 75%. However, the majority of patients are not eligible for an organ transplant or never receive one. Another option for the small number of patients without existing cirrhosis of the liver is surgical resection of the affected area. This yields a 70% 5-year survival rate, but carries a high risk of relapse. When surgery is not possible in advanced cases of HCC, it is treated with radiofrequency ablation and kinase inhibitor drugs such as sorafenib, which can extend the life expectancy of the patient but are rarely curative (Raza & Sood, 2014). Therefore, it is imperative to find novel druggable targets to improve treatment options for HCC as its prevalence continues to grow.

In the US, 54.9%, 16.4%, 14.1%, and 9.5% of HCC cases are related to hepatitis C infection, alcoholic liver disease, non-alcoholic fatty liver disease, and hepatitis B infection, respectively (Kanda et al., 2020). These different diseases can all produce chronic liver inflammation that leads to cirrhosis, which is the primary underlying risk factor for the development of HCC (Margini & Dufour, 2016). Non-alcoholic fatty liver disease (NAFLD) is characterized by a buildup of excess fat in the liver and is often caused by the accumulation of excess free fatty acids associated with obesity and other metabolic diseases. The chronic inflammation caused by NAFLD can progress to non-alcoholic steatohepatitis (NASH), which is a subtype of NAFLD involving apoptosis of hepatocytes and generation of scar tissue. NASH is essentially a more advanced stage of NAFLD and is therefore more frequently associated with the onset of HCC. The incidence of HCC in persons with NASH (5.29/1000 person-years) is more than 10 times higher than in those with NAFLD (0.44/1000 person-years) (Negro, 2020).

Due largely to its related comorbidities, obesity itself is a major risk factor for HCC development. Obesity (BMI>30) increases the relative risk of HCC development by 1.9 (Margini & Dufour, 2016). While the association between obesity and HCC is clear, the specific mechanisms behind this association are not completely known and it is likely the result of many overlapping factors in lipid metabolic dysregulation. Obesity results in increased levels of leptin, which contributes to the chronic inflammation that causes scar tissue formation, and can also activate cell cycle pathways to promote the proliferation of cancerous cells (Saxena et al., 2007). Obesity also results in increased fatty acid availability within the liver. This increased availability of fat in the liver could fuel the growth of HCC cells by supplying them with excess energy and resources needed for proliferation. HCC cells demonstrate an increased rate of fatty acid oxidation compared to normal cells (Patterson et al., 2011), demonstrating that they could rely on fatty acid

oxidation to fuel their growth. Obesity and Type 2 diabetes are also associated with insulin resistance, which results in excess free fatty acids. This excess fat accumulates in the liver and contributes to cirrhosis associated with NAFLD and NASH, which can therefore contribute to the onset or progression of HCC.

Genetic mutations are also frequently associated with the development of HCC. According to a study of 88 primary tumors isolated from HCC patients, the two main oncogenic pathways in HCC affected by genetic mutations are the Wnt/beta-catenin pathway and the JAK/STAT pathway. Both of these pathways are crucial for cell cycle control and are not commonly mutated in other types of cancer (Kan et al., 2013). Beta-catenin is a substrate that activates transcription of genes associated with survival and proliferation of cells. Wnt proteins are a family of glycoproteins that bind to receptors in the outside of cells to prevent the degradation of beta-catenin (Pai et al., 2017). Mutations in genes encoding beta-catenin or Wnts can result in the buildup of beta-catenin and transcription of survival genes, supporting cell proliferation and the progression of cancer development. As many as 90% of HCC cases have some mutation in the Wnt pathway, many of which are in the CTNNB1 gene, encoding beta-catenin (Liu et al., 2016). This gene is modified in certain cell lines, including SNU-398 (used in this study) in order to induce an HCC phenotype of excessive cell division and liver overgrowth.

Lipid Metabolism in Cancer

It has been long appreciated that the cellular changes that occur in carcinogenesis include significant metabolic alterations required to support growth and proliferation. Less appreciated is the fact that alterations in lipid metabolism is are very common and associated with carcinogenesis across tumor types (Snaebjornsson et al., 2020). Fatty acids (FAs) are the building blocks of biological lipids and play many critical roles within cells, including membrane construction, formation of signaling molecules, and energy storage. In normal adult tissue, FA synthesis is limited to the liver, adipocytes, and lactating breast, however cancer cells often activate de novo FA synthesis pathways to meet their needs. FAs are critical in every step of oncogenesis, from tumor initiation to metastasis all the way to development of drug resistance (Röhrig & Schulze, 2016).

HCC is no exception, often expressing a lipid metabolic phenotype that is highly altered relative to normal cells. Like many cancers, HCC tumor initiation and progression has been associated with increased rates of FA synthesis through the upregulation of enzymes including fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), ATP-citrate lyase (ACLY), and sterol-CoA desaturase (SCD1) (Sangineto et al., 2020). Sterol regulatory binding protein 1 (SREBP1) is a major regulator of these lipogenesis pathways, and its expression has been found to be upregulated in HCC (Yamashita et al., 2009). In addition, high levels of SREBP1 in primary tumors obtained from HCC patients was significantly correlated to a high risk of mortality, lower survival, and overall poorer prognosis in a study of 54 tumor samples by Yamashita and colleagues. This evidence demonstrates the importance of FA synthesis upregulation in the progression of HCC.

Oxidation of free FAs is also an important part of cancer metabolism because it supplies cells with energy in the form of ATP and NADPH (Qu et al., 2016). Normally, FA synthesis and oxidation cannot occur concurrently because the enzymatic pathways are unidirectional. However, cancer cells display a greater metabolic plasticity than normal cells and this allows them to adjust their rates of FA synthesis and oxidation based on the relative concentrations of metabolites like acetyl-CoA (Carracedo et al., 2013). This metabolic plasticity of cancer cells is ultimately what enables them to thrive in a range of conditions, allowing for tumor growth, progression, and metastasis. Inhibition of FA oxidation holds promise as a potential cancer therapy because it would limit the metabolic options available for cancer cells to use, and therefore limit the range of environments in which they could thrive. Carnitine palmitoyl transferase 1 (CPT1) is the first and rate limiting step of fatty acid oxidation. The upregulation of CPT1 has been implicated in a number of cancers, and its pharmacological inhibition has been shown to decrease the viability of cancerous cell lines (Melone et al., 2018). This evidence demonstrates how cancer cells rely on FA oxidation to support their proliferation and indicates that it is a pathway of interest in the development of novel cancer therapeutic strategies.

Drug Screen

Based on the evidence that HCC relies on upregulated rates of lipid synthesis and oxidation to support its proliferation, we hypothesize that inhibiting enzymes involved in these pathways will decrease the proliferation of HCC cells. To test this hypothesis, we built a library of 187 compounds targeting lipid metabolic enzymes and pathways to be screened in human HCC liver cells. Enzymes and compounds of interest were chosen based on a literature review of previous work in the field. Many of these compounds are used to treat metabolic disorders, including heart disease, Type 2 diabetes, obesity, and NASH. Others are preclinical compounds, laboratory grade enzyme inhibitors, or failed clinical candidates for these indications. Following drug exposure, the relative proliferation of the cells was assessed, and 'hit' compounds were identified as those having the greatest degree of anti-proliferative effects on the cells. Select hit compounds were further investigated to determine the dose-dependency of their anti-proliferation effects. The preliminary screens and dose response screen identified two primary compounds of interest, which merited further study. Mass spectrometry was used to identify metabolites produced by treated and untreated cells to further elucidate the mechanisms by which these compounds negatively affect cell proliferation.

In a collaboration with the Evason lab at the University of Utah, the drug library is also being screened in a zebrafish HCC model to determine how these compounds affect tumor growth in vivo. The zebrafish have a liver specific genetic mutation in their beta catenin gene that causes a liver overgrowth phenotype present in larval zebrafish that eventually leads to the development of HCC in these animals. The degree of hepatic overgrowth relative to an untreated control is used to show how compounds can reduce this liver overgrowth phenotype. The fish are treated as larvae, when they are transparent, and the size of their livers can be monitored and used as a proxy to represent HCC

development. Hit compounds are those that limit the degree of overgrowth in larval zebrafish. This portion of the project is still a work in progress, but its results will be used to inform our future endeavors.

The overall goal of this project is to complete a broad survey of lipid metabolism in HCC to identify possible new therapeutic targets and to investigate how HCC uses lipid metabolism to accelerate its progression. I hypothesize that inhibiting rate-limiting enzymes in lipid synthesis and oxidation will produce the greatest anti-proliferative effect on the HCC cells because in this condition, they will be unable to extract energy and carbon from lipids to fuel their growth.

METHODS

Library design and purchase:

Target enzymes were identified through a literature review of prior studies examining the variation in enzyme associated with cancer. Potential drugs to target these enzymes were identified using web-based research and a review of current therapeutics in use for the treatment of metabolic disorders. Preclinical and failed clinical trial candidates were also considered. A lipid targeting compound library containing 182 compounds was selected from a larger metabolism focused library available from a commercial vendor (Medchem Express) and 5 auxiliary compounds were purchased to augment the range of enzymatic targets represented within the library. Detailed library information is available upon request.

Cell culture:

HUH7, SNU-398, and HepG2 cells were obtained from American Type Culture Collection (ATCC) and grown in Dubelccos's Modified Eagle Medium supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin/streptomycin antibiotic (ATCC). They were cultured in accordance with ATCC protocol and passaged weekly throughout the duration of the experiments. They were stored in a 37° C 5% CO_2 incubator and maintained in 10 cm plates prior to screening.

Drugs and dilution:

All compounds were dissolved in DMSO, except for Lp-PLA2-IN1, Hydroxycholesterol, Rovalozac, Fluvastatin, and BAR502, which were dissolved in ethanol, ethanol, ethanol, water, and DMF respectively at a concentration of 10 mM. Compounds were sealed and stored at -80° C when not in use. Two dilutions were prepared: a high dose of 1 mM and a low dose of 0.1 mM. These solutions were added to media at a 1:100 ratio for cell exposure, resulting final concentrations of 10 µM and 1µM.

Screening:

Cells were harvested and transferred to 96 well assay plates at a density of 5000 cells per well 24 hours prior to the start of drug exposure. For HUH7 and HepG2 adherent cells, 100 µL of standard media was replaced with 100 µL of media with drug solution. For SNU398 semi adherent cells, drugs were added to media at a 10-fold higher concentration and 10 µL drug media solution was added to 90 µL standard media. Cells were stored in the 37° C 5% CO_2 incubator during treatment. 3 replicates of each treatment condition were completed.

Following drug exposure, cells were incubated for 5 days before being counted. The drug library was divided into 3 plates, with 2 dilutions of each plate. Each dilution was tested 3 times in each cell line. Following the 5-day incubation period, an Alamar Blue Assay was used to assess the relative proliferation of the cells. Resazurin sodium salt was dissolved in sterile water at a concentration of 0.3 mg/mL. It was applied to cells at a 1:10 dye:media concentration and allowed to incubate for 1 hour and 40 minutes prior to plate reading. Plates were read using a BioTek Synergy Neo2 reader for fluorescence with excitation at 560 and emission at 590 nm.

Dose Response:

Dose response screens were completed in HUH7, SNU398, and HepG2 cell lines. Compounds were selected based on results of the initial screen and tested at concentrations of 10 µM, 3 uM, 1 µM, .3 µM, and .1 µM. Seeding, exposure, and proliferation assays were completed in the same way as the initial screen.

Mass Spectrometry Sample Preparation:

HUH7 cells were seeded at a density of 5.0×10^5 in 6 cm plates for drug treatment samples and 4.5×10^5 6 cm plates for controls. They were incubated for 48 hours prior to drug treatment to allow adherence. Drugs PF-3845 and PF-04457845 were added to standard media at a concentration of 3.3 µM. For treatment, media was fully replaced with 4 mL drug containing or control media and cells were incubated for another 48 hours prior to extraction. 4 plates were prepared for each condition (control, drug 1, and drug 2) to have 1 plate as a cell count and 3 replicates each.

For extraction, spent media was aspirated and cells were rinsed with 2 mL of phosphate buffered saline. 500 µL of ice-cold 80:20 methanol:H_2O was applied to each plate and they were stored on ice throughout the procedure. Cells were removed from plates with a Corning Cell Scraper and placed into clean Eppendorf tubes. The tubes were centrifuged at 13000 rpm for 10 minutes at 4 C, then supernatant was transferred to fresh tubes and 250 µL of chloroform was added to each. 150 µL of this final aqueous solution was placed into sample vials and stored in -80° C until use.

Data Analysis:

The plate reader reports a fluorescence value of the dye added to each well of the plate that is proportional to the total redox activity of the well, a proxy for total cell number. In order to evaluate the proliferation of cells in each well and to account for errors in plating as well as cell growth, the full 96-well plate was averaged, excluding the edges, and a standard deviation was computed for each well. A z-score was calculated for every well using this formula:

$$\frac{(Plate\ Average) - (Experimental\ Value)}{Plate\ Standard\ Deviation}$$

Three replicates of each plate were completed, and the z-score values were averaged to give a final z-score for each compound. Z-score scatterplots were generated by taking the average and standard deviation of z-score hits in each cell line to evaluate the efficacy of each hit relative to other hit compounds. All calculations were completed in Excel. Dose response curves were generated using a non-linear IC_{50} regression in Graphpad Prism version 8. The mass spectrometry glutathione graph was prepared using normalized ion counts as determined by previously collected standard using the analysis program MAVEN.

RESULTS

Library and Hit Distribution:

To evaluate the role of lipid metabolism in HCC development, we assembled a library of 187 lipid metabolic compounds to be screened in HCC cell lines HUH7 and SNU398, as well as in zebrafish in collaboration with another lab. Figure 1 shows the top 20 general enzyme classes targeted by the library. It was intended to test a broad range of lipid metabolism, from fatty acid synthesis and oxidation to the synthesis of signaling molecules like hormones. The top general target of the library is the cytochrome P450 (CP450) enzyme family, which includes a broad range of enzymes involved primarily in metabolism and signaling molecule synthesis. The following two greatest target classes were autophagy and phospholipase enzymes. Like the CP450 enzyme family, autophagy is a broad category that encompasses many individual enzymes and signaling pathways.

For the purpose of this study, we define a hit compound as one that impacted the relative proliferation value by greater than one standard deviation compared to the plate average. Table 1 and Table 2 show the hit compounds, from greatest to least effect, in the HUH7 and SNU398 cell lines, respectively. Many of the most efficacious compounds belong to CP450, autophagy, and phospholipase general target classes.

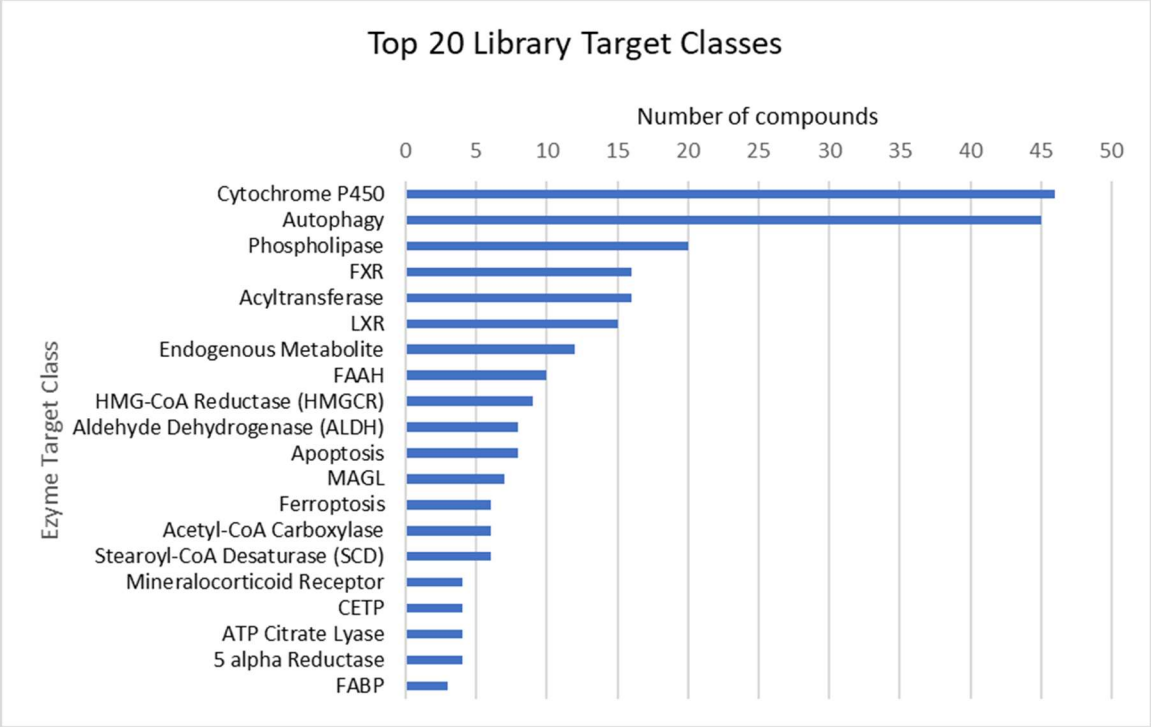


Figure 1: Bar graph showing the top 20 target enzyme classes of the compound library we assembled. The x axis shows the number of compounds per enzyme class on the y axis.

Table 1:

10 uM z-score	1 uM z-score	Compound	Target Class	Specific Target
-3.26847	-3.44999	Quinacrine	Autophagy; Mitophagy; Phospholipase	Inhibitor of phospholipase A2 enzyme
-3.09862	1.18741	3-Nitrocoumarin	Phospholipase	Inhibitor of phospholipase enzyme
-3.02733	-3.32422	Disulfiram	Aldehyde Dehydrogenase (ALDH);	Inhibitor of aldehyde dehydrogenase enzyme
-3.02458	-3.33619	Darapladib	Phospholipase	Inhibitor of phospholipase enzyme
-2.73745	-2.95551	Chrysoplenetin	CP450	Inhibitor of CYP3A4
-2.56138	0.92315	Guggulsterone	AKT, autophagy, FXR	Antagonist of farnesoid x nuclear transcription factor receptor
-2.46316	-2.45576	ML349	Phospholipase	Inhibitor of phospholipase enzyme
-2.34761	-1.74314	Alpha-Estradiol	5 alpha Reductase; Endogenous Metabolite	Biologically active synthetic estrogen
-2.27746	-2.56860	PF-3845	Autophagy; FAAH	Inhibitor of fatty acid amide hydrolase enzyme
-2.17067	-1.98725	TMS	Cytochrome P450	Inhibitor of CYP1B1
-2.08864	-2.19793	LY2183240	Autophagy; FAAH	Inhibitor of fatty acid amide hydrolase enzyme
-1.73834	-1.98497	DO-264	MAGL	Inhibitor of monoacylglycerol lyase enzyme
-1.59460	-1.72503	PF-04457845	Autophagy; FAAH	Inhibitor of fatty acid amide hydrolase enzyme
-1.37092	0.76036	DMU2105	Cytochrome P450	Inhibitor of CYP1B1 and less potently CYP1A1
-1.35395	-2.00040	KT182	MAGL	Inhibitor of monoacylglycerol lyase enzyme
-1.08937	-1.20121	Lp-PLA2-IN-3	Phospholipase	Inhibitor of phospholipase enzyme
-1.07912	-0.37182	AZ876	LXR	Agonist of liver x nuclear transcription factor receptor
-1.05099	0.39746	Seviteronel	Cytochrome P450	Inhibitor of CYP17A1
-1.03033	-1.73920	4'-Methylchrysoeriol	Cytochrome P450	Inhibitor of CYP1B1
-1.01007	0.84497	Dafadine-A	Cytochrome P450	Inhibitor of DAF-9 CP450 enzyme
-0.99364	-1.11819	GSK1940029	Stearoyl-CoA Desaturase (SCD)	Inhibitor of Steroyl-CoA Desaturase enzyme
-0.96001	-0.65406	Abiraterone	CP450	Inhibitor of CYP17A1
-0.65049	-1.59643	F1P1	Autophagy, phospholipase	Inhibitor of phospholipase enzyme
-0.44318	-1.47136	DMU2139	Cytochrome P450	Inhibitor of CYP1B1

Tables 1 & 2: Hit compounds from the full library in the HUH7 (Table 1) and SNU398 (Table 2) cell lines. Z-scores for high (10 uM) and low (1 uM) doses were calculated using the average and standard deviation of each 96-well plate tested. Target class is the general target as described in the library whereas specific target describes the primary enzyme targeted by each compound. Compounds are in order of decreasing anti-proliferative effect.

Table 2:

10 uM z-score	1 uM z-score	Compound	Target Class	Specific target
-1.72432	0.28657	3-Nitrocoumarin	Phospholipase	Inhibitor of phospholipase enzyme
-1.61653	-1.10183	Quinacrine	Autophagy; Mitophagy; Phospholipase	Inhibitor of cholinesterase
-1.60368	-0.91260	Darapladib	Phospholipase	Inhibitor of phospholipase enzyme
-1.60337	-1.33884	Pitavastatin (Calcium)	Apoptosis; Autophagy; HMG-CoA Reductase	Inhibitor of hydroxy-methylglutaryl-CoA
-1.58724	-3.11166	Disulfiram	Aldehyde Dehydrogenase (ALDH)	Inhibitor of aldehyde dehydrogenase enzyme
-1.56694	-2.26200	(3S,5S)-Atorvastatin	Cytochrome P450	Inhibitor of CYP2B6 and CYP3A
-1.40032	-0.70387	DMU2139	Cytochrome P450	Inhibitor of CYP1B1
-1.37844	-1.66180	DO-264	MAGL	Inhibitor of monoacylglycerol lyase enzyme
-1.24487	-0.22061	Alpha-Estradiol	5 alpha Reductase	Biologically active synthetic estrogen
-1.21567	-1.34683	DMU2105	Cytochrome P450	Inhibitor of CYP1B1 and less potently CYP1A1
-1.21443	-2.25895	PF-3845	Autophagy; FAAH	Inhibitor of fatty acid amide hydrolase enzyme
-1.18800	-0.28630	CM10	Aldehyde Dehydrogenase (ALDH)	Inhibitor of aldehyde dehydrogenase enzyme
-1.18090	0.87991	LCL521 dihydrochloride	Phospholipase	Inhibitor of phospholipase enzyme
-1.15568	-0.32439	Esaxerenone	Mineralocorticoid Receptor	Antagonist of mineralocorticoid receptor
-1.09534	0.76402	Rovazolac	LXR	Agonist of liver x nuclear transcription factor receptor
-1.08118	-0.75050	Lovastatin	Autophagy; Ferroptosis	Inhibitor of hydroxy-methylglutaryl-CoA
-1.07360	-1.58657	Atorvastatin	Autophagy; Ferroptosis	Inhibitor of hydroxy-methylglutaryl-CoA
-1.07310	-0.37703	CP-640186 (hydrochloride)	Acetyl-CoA Carboxylase	Inhibitor of acetyl-CoA Carboxylase
-1.05999	-1.28793	Xanthohumol	Acyltransferase	Inhibitor of acyltransferase enzyme
-1.01516	-0.18326	GW3965 (hydrochloride)	LXR	Agonist of liver x nuclear transcription factor receptor
-0.80588	-0.71435	Chrysoplenetin	Cytochrome P450; Ferroptosis; P-glycoprotein	Inhibitor of CYP3A4
-0.80491	-1.14074	Canrenone	Endogenous Metabolite	Inhibitor of steroid synthesis enzymes
-0.74554	-0.84148	PF-04457845	autophagy faah	Inhibitor of fatty acid amide hydrolase enzyme
-0.68491	-1.10362	ML349	Phospholipase	Inhibitor of phospholipase enzyme

Figure 2 shows these results graphed in a scatterplot of high versus low dose z-scores in each cell line. These z-scores were calculated relative to other hit compounds, so these graphs show the relative strength of each hit compound as compared to others. Hit compounds in the HUH7 cells showed a high degree of correlation between high and low doses, as indicated by the linear relationship between the two axes. Compounds in the lower left quadrant, including Quinacrine, Disulfiram, Darapladib, Chrysoplenein, and PF-3845, with the most negative z-scores, are the most effective and consistent across high and low doses. In the upper right quadrant, 3-Nitrocoumarin and Guggulsterone show a strong antiproliferative effect at a high dose, but not the low dose. This means they have a dose-dependent effect within the tested range.

The SNU cell line had more variability in its proliferation than the HUH7s, resulting in more noisy data and a less clear correlation between high and low doses. That said, many of the more efficacious compounds including Disulfiram, Quinacrine, and Darapladib had a strong negative effect in both cell lines. In addition, 3-Nitrocoumarin produced the same high-dose dependent effect in the SNU398s as observed in the HUH7s. This demonstrates that even though the SNU398 cell line produced noisier data, the overall effect of many of the compounds were reproducible.

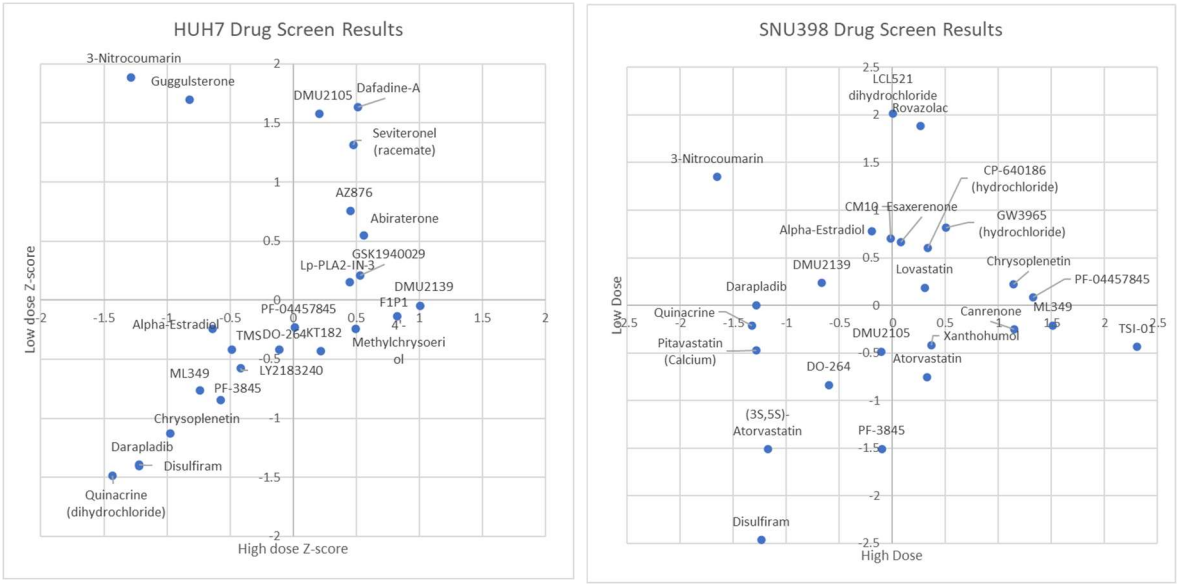


Figure 2: Scatterplots showing the high versus low dose z-scores of hit compounds in HUH7 and SNU398 cell lines, respectively. Z-scores were calculated using the average and standard deviation of the hit compound z-scores in to evaluate their efficacy relative to the other hits.

The general classes of hit compounds were fairly representative of the overall library distribution, with CP450, autophagy, and phospholipase inhibitors as the top 3 enzymatic targets (Figure 3). There is a dramatic difference between these top 3 classes and the next class, fatty acid amide hydrolase (FAAH), suggesting that the top 3 classes may be more cytotoxic than the others. It is unclear whether this effect is cancer cell specific or common across all cell types. Nonetheless, these results highlight which general enzyme targets may be most important for the proliferation of HCC cells.

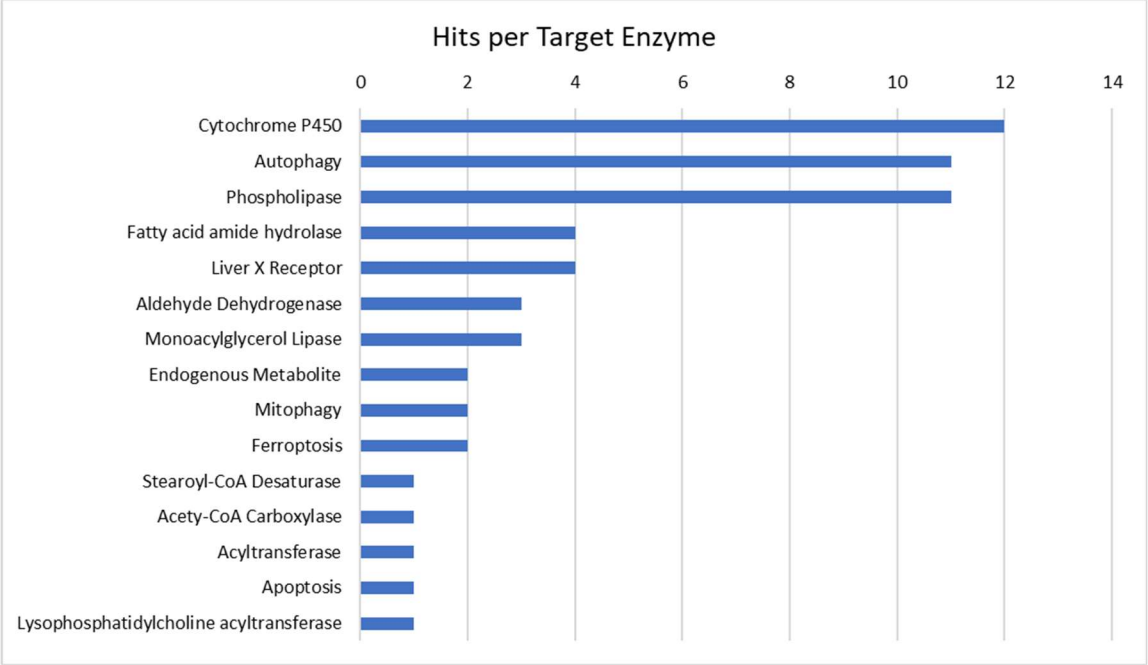


Figure 3: Number of hit compounds from both HUH7 and SNU398 cell lines graphed according to their general enzymatic target class

In order to compare hit compounds between HUH7 and SNU398 cells, we graphed the high dose z-score of each hit in both cell lines, calculated relative to the other hits in each cell line (Figure 4). The most efficacious compounds, in the lower left quadrant, showed a correlation between both cell lines. These include Quinacrine, 3-Nitrocoumarin, PF-3845, Disulfiram, and Alpha Estradiol. As expected from the results shown in Figure 3, these compounds have general targets of phospholipase, autophagy, and CP450 enzymes.

We also found that some compounds exhibit a cell type-specific effect, where they reduce proliferation in one cell line but not the other. The lower right quadrant of the graph contains compounds that negatively affected SNU398 cells but not HUH7 cells. We found that statin compounds, including Pitavastatin, Atorvastatin, and Lovastatin fall into this category. Statins inhibit HMG-CoA reductase, which is involved in cholesterol

biosynthesis and autophagy pathways. Conversely, some CP450 inhibitors including Abiraterone and Seviteronel had a strong positive effect on proliferation of SNU398 cells and little to no effect in HUH7 cells. These compounds target CP450 enzymes involved in the synthesis of hormones, suggesting that SNU398 cells may be more susceptible to these specific signaling molecule directed effects.

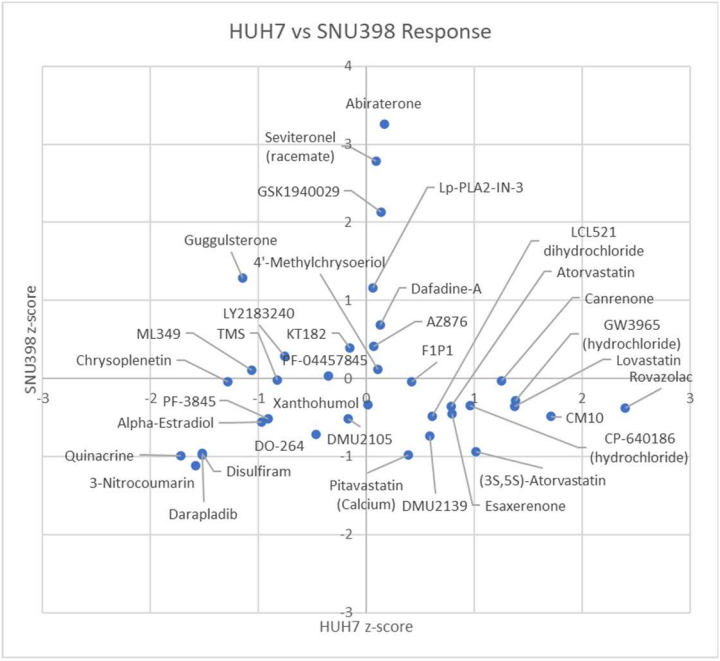


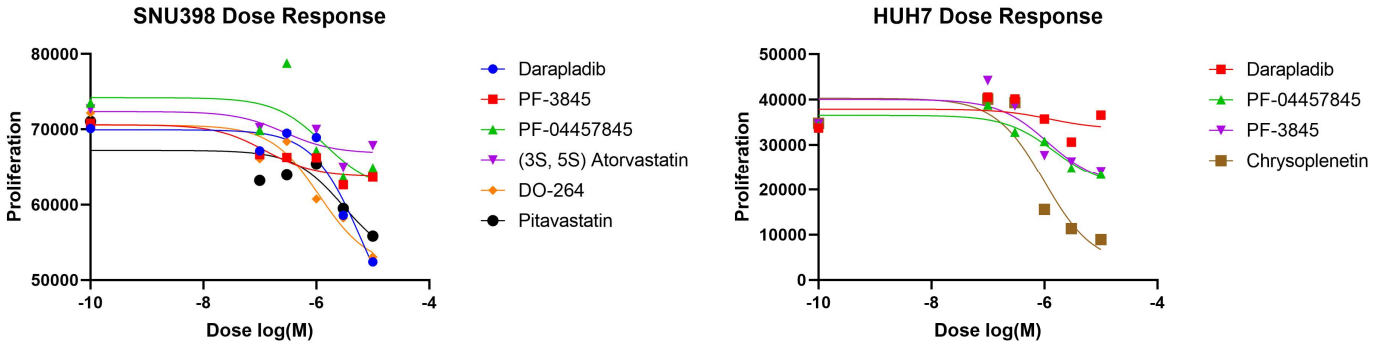
Figure 4: Scatterplot comparing hit compounds in HUH7 versus SNU398 cell lines based on high dose z-score. Z-scores for this graph were calculated relative to the high dose hit z-scores in each cell line.

Dose Response Analysis:

Based on the results of the full library screen in both cell lines, we chose compounds for dose response analysis in HUH7, SNU398, and a third cell line HepG2. The results of this analysis are shown in Figure 5. In the HUH7 cell line, Chrysoplenetin, a CP450 inhibitor, produced a strong anti-proliferative effect at the high dose of 10 uM whereas compounds PF-3845 and PF-04457845, FAAH inhibitors, produced a more moderate effect. The same moderate effect of the FAAH inhibitors was observed in the SNU398 cell line, where Darapladib, DO-264, and Pitavastatin generated a much stronger effect similar to Chrysoplenetin in the HUH7s. We are most interested in the moderate proliferation limiting compounds as a potential therapeutic because they are less likely to be cytotoxic than those compounds with a very strong effect.

DRUG	HUH7	SNU398	HEPG2
Atorvastatin	NA	~ 0.03141	7.57E-08
(3S, 5S) Atorvastatin	NA	2.767E-07	2.24E-07
Chrysoplenetin	9.99E-07	NA	NA
Darapladib	1.25E-06	6.503E-06	~ 0.03339
DMU2105	NA	2.026E-13	2.58E-07
DO-264	NA	1.153E-06	9.01E-06
LY-2183240	~ 0.02623	NA	NA
PF-04457845	1.48E-06	1.373E-06	4.91E-09
PF-3845	1.13E-06	1.229E-07	6.27E-08
Pitavastatin	NA	3.032E-06	1.22E-07
Quinacrine	~ 0.05354	2.342E-08	3.14E-06
Torcetrapib	3.8E-07	NA	NA

Table 3: Calculated IC50 values from each compound tested in each cell line. NA means the compound was not tested in that cell line and grey text means the IC50 value was outside of the experimental range (10e-8 M to 10e-5 M). IC50 values within experimental range are in black text and the color of each cell corresponds to the relative strength of that compound compared with the others.



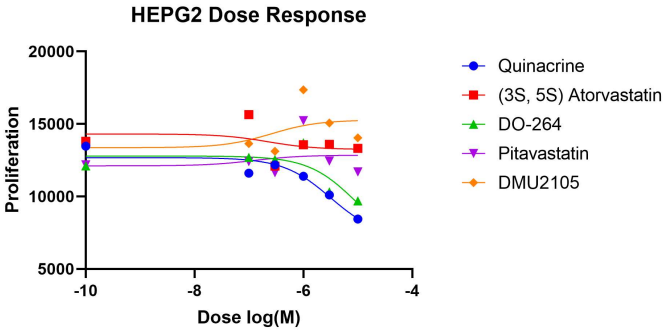


Figure 5: Dose response curves generated for HUH7, SNU398, and HepG2 cell lines using non-linear regression curve fitting. Graphs show only compounds that produced an IC50 value within the experimental range of .1 to 10 uM.

Table 3 shows the calculated IC_{50} values of all compounds tested in the three cell lines. Some compounds did not produce an IC_{50} within the experimental range, and these values are shown in grey text on the graph. This means that the compounds were not effective at the doses we tested and shows that their anti-proliferative effects in the original screen may not be reproducible. PF-04457845 and PF-3845 produced IC_{50} values within experimental range in both cell lines, which informed our choice of using these compounds to treat cells for mass spectrometry metabolite analysis later in the project.

In Vivo Screening:

As mentioned previously, this library of compounds is also being screened in zebrafish *in vivo* as a collaboration with the lab of Dr. Kim Evason. The fish being used have a mutation in their beta-catenin gene which results in a liver overgrowth phenotype consistent with the one seen in HCC. Figure 6 shows our results from this study as hit compounds in each of the three model systems as a Venn diagram. The compounds that have an effect on all 3 systems – 3-Nitrocoumarin, PF-3845, and DO-264 - are of most interest to us because they are effective in vitro as well as in vivo. This is promising as a potential cancer therapeutic compound. Compounds effective in two systems are also of interest and these results will be used to inform the future inquiries of this project.

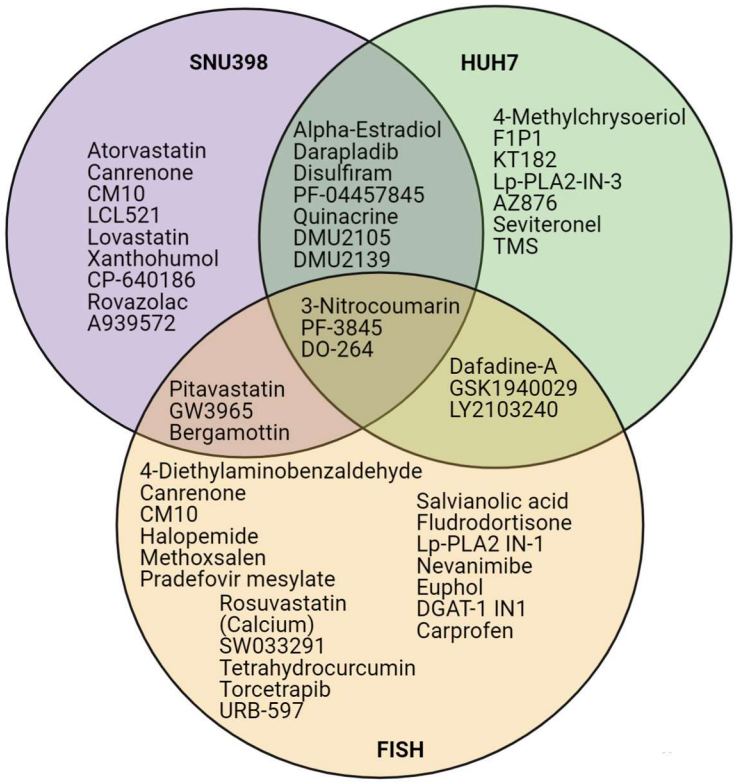


Figure 6: Venn diagram showing overlap of hit compound in HUH7 cells, SNU398 cells, and zebrafish with a beta-catenin driven liver overgrowth phenotype. 160 out of 187 compounds have been tested in all three models so far.

Mass Spectrometry:

Using results from the full compound screen, dose response curves, and fish screen, we identified two FAAH inhibitors, PF-3845 and PF-04457845, whose mechanism of action we wished to elucidate further. Eventually, we will follow up on more compounds, however these two were chosen for the initial analysis because PF-3845 had an effect in all three systems tested, indicating that FAAH may be a promising enzymatic target class. We used liquid chromatography mass spectrometry (LCMS) to evaluate metabolite abundances in treated versus untreated HUH7 cells. The main finding of this analysis was that glutathione levels were highly upregulated in treated versus untreated cells, as shown in Figure 7. Elevated glutathione could be indicative of a higher level of oxidative stress in the treated cells.

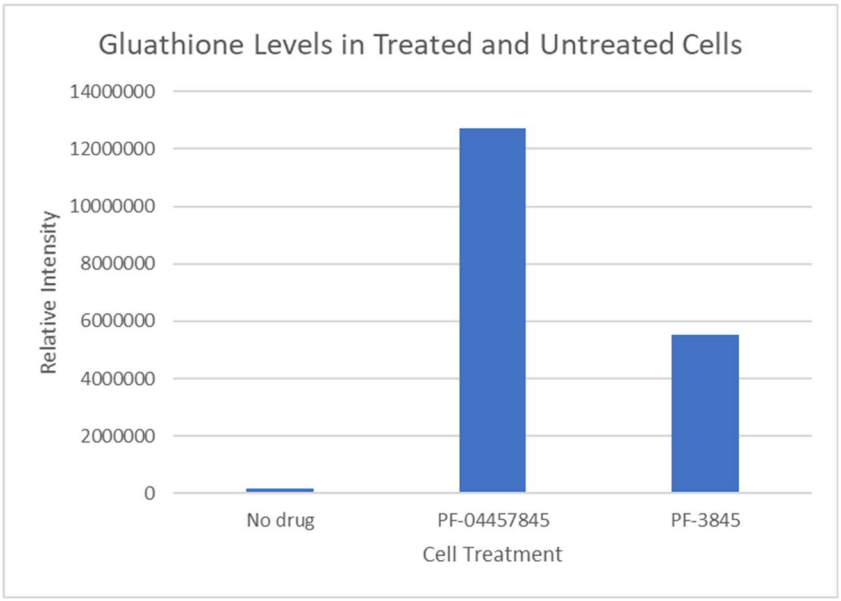


Figure 7: Bar graph showing relative quantities of glutathione in HUH7 cells treated with no drug or FAAH inhibitors PF-04457845 and PF-3845 as determined using mass spectrometry.

DISCUSSION

General findings:

We hypothesized that HCC cells were using lipid synthesis and oxidation to obtain energy and carbon to fuel their accelerated rate of growth. Therefore, we thought that inhibiting the primary rate-limiting enzymes in these pathways, including fatty acid synthase, carnitine palmitoyl transferase, and sterol regulatory binding protein, would have the greatest anti-proliferative effect on the cells because this inhibition would prevent the cells from using lipids for energy. Instead, we found that the most effective anti-proliferative compounds were those targeting CP450, autophagy, and phospholipase enzyme families, which are not involved in the primary steps of lipid synthesis and oxidation. Rather, these enzyme families are primarily involved in the synthesis of lipid-based signaling molecules, including hormones and sterols. This supports our hypothesis that lipid synthesis and oxidation enzymes are important for the proliferation of HCC cells and may represent a new therapeutic target for HCC, but possibly not for the reasons we initially thought.

The top target classes represented by our library are CP450, autophagy, and phospholipase enzyme families. The CP450 family includes a broad range of enzymes involved in hormone and androgen synthesis as well as drug metabolism. Like the CP450 enzyme family, autophagy is a broad target family that encompasses many individual enzymes and signaling pathways. Therefore, many of the compounds we tested target CP450 and autophagy enzymes in addition to their primary enzymatic target. The same can be said for phospholipase enzymes. The following top represented enzyme target classes are those more traditionally thought of as lipid metabolism, including acyltransferase, FAAH, and HMG-CoA reductase enzyme families.

Cell Line Results:

In the HUH7 cell line, the top 5 hits were Quinacrine, 3-Nitrocoumarin, Disulfiram, Darapladib, and Chrysoplenetin targeting phospholipase, aldehyde dehydrogenase, and CP450 enzyme classes. Quinacrine, 3-Nitrocoumarin, and Darapladib all inhibit the phospholipase A2 (PLA2) enzyme, which cleaves phospholipids to release arachidonic acid (Kudo & Murakami, 2002). Arachidonic acid is a lipid species that is used to produce prostaglandins, an important class of signaling molecule. Therefore, the inhibition of the PLA2 enzyme could limit downstream cell survival signaling pathways in treated cells. Disulfiram is a drug commercially known as Antabuse, which inhibits aldehyde dehydrogenase and prevents the metabolism of alcohol in the liver. It has been previously noted that Disulfiram can have an anti-cancer effect (Ekinci et al., 2019), however its exact mechanism is unclear. Chrysoplenetin inhibits the CP450 enzyme CP3A4, which is primarily responsible for drug metabolism in the liver.

The top four hits in the SNU398 cell line were the same as observed in HUH7; 3-Nitrocoumarin, Quinacrine, Darapladib, and Disulfiram. Chrysoplenetin did not have a strong effect in this cell line. The next top three hits in the SNU398 cell line were

Pitavastatin, which inhibits hydroxy-methylglutaryl-CoA reductase (HMG-CoA), and (3S,5S)-Atorvastatin and DMU2139, which both inhibit enzymes from the CP450 family. HMG-CoA is the rate limiting enzyme involved in cholesterol biosynthesis, so most statin drugs used to treat high cholesterol target this enzyme. (3S,5S)-Atorvastatin inhibits enzymes CYP2B6 and CYP3A, both involved in drug metabolism. DMU2139 is an inhibitor of CYP1B1, whose exact function is unknown. However, mutations in this gene have been associated with the development of glaucoma, so it is thought that the enzyme may be involved in the synthesis or reception of a signaling molecule involved in cell development (CYP1B1 Gene - NCBI, 2021).

The distribution of hit compounds we found were generally representative of the overall library distribution. The top four target enzyme classes of hit compounds were CP450, autophagy, and phospholipase inhibitors, which are the same top classes as the full library. The following classes include FAAH and Liver X Receptor inhibitors. All five of these top classes have a shared theme: involvement in signaling molecule synthesis and reception pathways.

Comparing the hit compounds from each cell line highlighted some possible genotype specific effects. Statin compounds had a greater negative growth effect on the SNU398 cells than on the HUH7 cells. These compounds are used to treat high cholesterol, and do so by inhibiting HMG-CoA reductase. Cholesterol has many functions within a cell as a signaling molecule, as demonstrated by the large quantity of cholesterol receptors embedded in cellular membranes. One known function of cholesterol signaling is the activation of the canonical Wnt cell survival signaling pathway (Sheng et al., 2014). SNU398 cells have a genetic mutation in their CTNNB1 gene, which produces an excess of beta-catenin and progresses cell survival via the Wnt pathway. This could explain why statin compounds showed a greater antiproliferative effect in the SNU398 cell line that the HUH7 cell line. By inhibiting cholesterol production, they may limit cholesterol-based activation of the Wnt signaling pathway, which is the primary driver of cell proliferation in the SNU398 cell line. This information could be useful in directing treatment for cases of HCC that are associated with a CTNNB1 mutation.

Dose Response:

The results of our dose-response analysis highlighted some compounds that were highly cytotoxic, including Chrysoplenetin, Pitavastatin, and DO-264. On the other hand, we also found compounds that produced a less dramatic proliferation-limiting effect, including PF-3845 and PF-04457845. In searching for a cancer therapeutic, we are more interested in those less dramatic proliferation limiting compounds because the goal is not to kill cells – it is to limit the growth of cancerous cells. Therefore, PF-3845 and PF-04457845 are the two compounds we chose for our initial follow-up tests. Ideally, we would have tested these compounds in a normal cell line as well as the cancerous cell lines to determine if the observed effects are cancer cell specific. However, due to time constraints, that was not possible for this project.

Though we are lacking a normal cell control for in vitro results, we do have some in vivo results from our zebrafish collaboration project. The full library has not yet been completed, but we do have qualitative results from 160 out of 187 total compounds. Thus far, we have identified three compounds that have an anti-proliferative effect in all three model systems tested, which belong to the phospholipase and FAAH inhibitor general enzyme classes. This reinforces our in vitro results and suggests that these pathways are important for HCC development in vivo.

Glutathione Upregulation:

Based on our cell screen, dose response, and fish results, we chose to investigate FAAH inhibitors PF-3845 and PF-04457845 further using mass spectrometry to evaluate metabolites produced by treated versus untreated cells. The most significant result of this screen is that glutathione was highly upregulated in the treated cells compared to the normal control. Glutathione is produced by cells in response to oxidative stress, as it can scavenge and neutralize reactive oxygen species that lead to DNA damage and cell death. The primary function of FAAH is to break down anandamide into arachidonic acid and ethanolamine, which are endocannabinoid molecules used in downstream cell survival signaling pathways. It has been previously shown by Siegmund, *et al*. in 2006 that the buildup of anandamide can lead to the formation of reactive oxygen species, oxidative stress, and eventually cell death. Therefore, we propose that the inhibition of FAAH leads to glutathione upregulation in hepatocytes via the following mechanism:

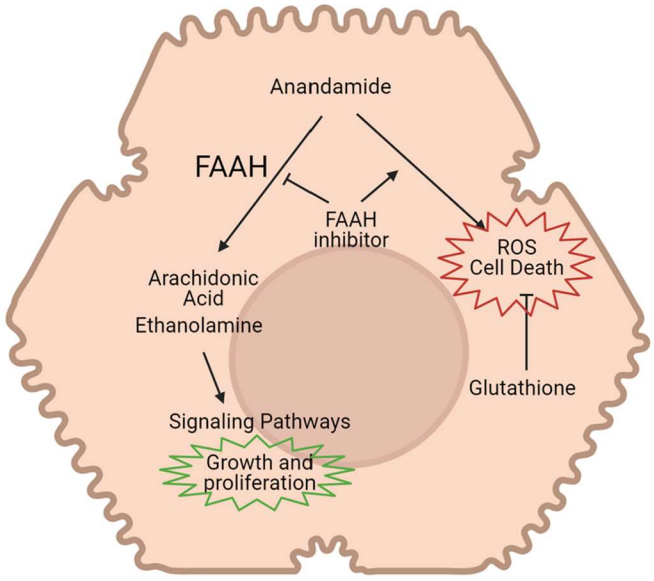


Figure 8: Schematic illustration showing the proposed mechanism for glutathione upregulation due to FAAH inhibition.

This is an important result in the context of a search for a cancer therapeutic because cancer cells are more susceptible to oxidative stress than normal cells are. Therefore, by increasing oxidative stress through inhibition of an enzyme like FAAH, we

could potentially selectively target cancer cells and slow their growth while leaving healthy cells unaffected. In addition, these findings again demonstrate the importance of lipid-based signaling molecules. Anandamide, arachidonic acid, and ethanolamine are all used by signaling pathways within the cell to mediate growth and proliferation. Manipulating the synthesis of these metabolites through inhibition of FAAH resulted in a decrease in HCC cell proliferation, highlighting their importance.

CONCLUSION

This study demonstrates that the upregulation of lipid metabolic enzymes in HCC cells could be due to increased reliance on signaling pathways that use lipid-based signaling molecules to promote cell proliferation. Therefore, signaling molecule synthesis and use within cells could represent a new therapeutic target for the treatment of HCC. The specific signaling pathways and molecules used to progress proliferation in HCC cells remain mysterious and merit further investigation in the future of this project.

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