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Characterizing the Effects of Takeda's Inhibitor on the SREBP Family of Transcription Factors

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Sterol regulatory element-binding proteins (SREBPs) are a set of transcription factors that bind to the SRE element. This element is involved in regulating expression of genes involved in cholesterol and fatty acid synthesis as well as the SREBPs themselves. This could make them a valuable drug target for treating non-alcoholic steatohepatitis (NASH), the third largest cause of liver transplants in the US. Takeda developed a series of compounds that were able to selectively inhibit SREBPs as an off target effect through an unknown mechanism. To examine this effect a dual luciferase assay was used to measure the expression of wild-type and mutant SREBP promoters when treated with these compounds. It was found that these compounds equally inhibit all SREBP promoters, regardless of the presence or absence of SRE elements. This suggests that these compounds inhibit SREBP expression through some shared regulatory element that is not the SRE.

Dedication

To my parents Dan and Rose Haddad for always being there for me and supporting me in my education.

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Chapter 1

Introduction

This section discusses background information for the thesis. It starts with general information about non-alcoholic fatty liver disease (NAFLD) and NASH. Then it describes the known effects of the Takeda compounds. Finally it describes known information about the regulation of SREBP expression and transport.

NAFLD and NASH

In the developed world about one third of people are estimated to have NAFLD (Machado 2014, Vernon 2011). Of these NAFLD cases about 15% will go on to develop NASH (Machado 2014, Vernon 2011). These diseases are the third most common reason for liver transplants in the United States (Machado 2014). They also contribute to the incidence of a cancer called hepatocellular carcinoma (Machado 2014). As these diseases are strongly associated with obesity, their incidence is expected to increase in the coming years (Machado 2014, Vernon 2011). This makes NAFLD and NASH a significant global health problem. Obesity is thought to promote NASH by driving insulin signaling and promoting inflammation (Machado 2014). Insulin signaling increases the synthesis of fatty acids in the liver (Chen 2004, Machado 2014). These fatty acids are toxic to liver cells at high enough concentrations, and together with inflammatory response lead to cell death and fibrosis (Angulo 2002, Machado 2014). This in turn leads to serious medical

complications that include cirrhosis and liver failure (Angulo 2002, Machado 2014). Unfortunately there is a significant unmet medical need for treatments that prevent or reverse NAFLD or NASH leaving transplant as the only effective treatment (Angulo 2002, Machado 2014).

Diagnosis and Treatment of NAFLD and NASH

Both NAFLD and NASH are largely asymptomatic, making detection of the disease difficult (Machado 2014). Diagnostic tools for testing NAFLD also have a number of limitations. Liver biopsies are considered the standard method of diagnosis, but this is invasive and is not recommended for general screening of populations (Angulo 2002, Machado 2014, Vernon 2011). Non-invasive methods have also been investigated like ultrasounds, computed tomography, and magnetic resonance spectroscopy (Machado 2014, Vernon 2011). However, these imaging methods have either low accuracy or high cost which prevents their widespread usage (Machado 2014). Currently there are no accepted treatments for NAFLD or NASH beyond exercise, diet changes, and weight loss (Machado 2014). While no drug has yet been approved for treatment, there are a few in clinical trials (Ratziu 2015). Currently, the best investigated drugs are those that are also used to treat diabetes (Machado 2014, Ratziu 2015). While diabetes is a risk factor for NAFLD and NASH, not all patients have it, limiting these drugs utility as a more general NASH treatment. Vitamin E has also been investigated for its protective effects, though the evidence so far has been inconclusive (Machado 2014, Ratziu 2015). The lack of an

approved treatment represents a significant unmet medical need that could be solved with new drugs targeting lipid metabolism.

Early Information on Takeda Compounds

A series of compounds were developed at Takeda Pharmaceuticals to inhibit Uba6 that were found to have interesting properties (Amidon 2014). Experiments analyzing the expression profiles of cells treated with a subset of these compounds showed that the expression of a modest number of genes (around 20 genes) were down-regulated (Takeda N.D). Most of these down-regulated genes are known to be positively regulated by a family of transcription factors called SREBPs (Takeda N.D.). Additional analysis by western blot and proteomic methods showed that after compound treatment the overall expression of these SREBPs (and several of the SREBP-dependent transcriptional targets) goes down, and that the amount of processed SREBP in the nucleus also was modestly reduced (Takeda N.D.). These effects appeared to be independent of the compounds inhibition of Uba6, as related molecules still potently inhibited Uba6 but did not modulate SREBP-dependent transcription (Takeda N.D.). Table 1 shows information on some of the Takeda compounds.

Table 1. Activities of the Takeda compounds. IC50 values and ability to inhibit SREBP expression of the Takeda compounds used in these experiments.

Compound	IC50 for Uba6	Inhibits SREBP expression
Lipid Inhibitor	>10 μM	Yes
Uba6 Inhibitor	0.014 μM	No
Dual Inhibitor	0.044 μM	Yes

As SREBPs are known to regulate fatty acid and cholesterol synthesis, a compound that selectively inhibits SREBP function could be potentially useful in treating NASH. It is currently unclear what the exact molecular target of this compound is and how the Takeda compound achieves these specific effects. Three of these compounds were selected for use in this thesis. A SREBP specific inhibitor, a Uba6 specific inhibitor, and one that inhibits both. Comparing the activity of these compounds should make it possible to isolate what effects are and are not caused by Uba6 inhibition.

The SREBP Gene Family

There are three SREBP proteins that are expressed from two genes (Chen 2004, Dif 2006, Fernandez 2008, Hirano 2001, Horton 2002, Peterson 2011, Xu 2013, Yellaturu 2009). All three are transcription factors that have three functional domains, a DNA binding domain that is related to the basic helix-loop-helix-leucine zipper family of transcription factors, a hydrophobic domain that anchors them to membranes, and a third domain that allows them to interact with other membrane bound proteins (Figure 1) (Horton 2002, Nohturfft 2000). SREBP1a and 1c are splice variants of the same gene,

with different first exons and different regulatory regions (Chen 2004, Dif 2006, Hirano 2001, Horton 2002, Peterson 2011, Xu 2013, Yellaturu 2009). SREBP2 is a separate but related gene (Chen 2004, Dif 2006, Hirano 2001, Horton 2002, Peterson 2011, Xu 2013, Yellaturu 2009). All SREBPs bind to the sterol response element (SRE) sequence, responsible for regulating the expression of genes involved in all aspects of lipid metabolism including fatty acid and cholesterol synthesis (Chen 2004, Sato 1996, Horton 2002, Xu 2013). While SREBPs have some overlapping functions it is generally held that SREBP1c regulates fatty acid-related metabolism, SREBP2 regulates cholesterol-related metabolism, and SREBP1a can affect transcription of virtually all lipid metabolism genes (Chen 2004, Horton 2002, Xu 2013). In liver cells SREBP1c is more highly expressed then SREBP2 while the reverse is true in some other cell types (Horton 2002, Xu 2013). SREBP1a is primarily expressed in cultured cell lines (Fernandez 2008, Xu 2013).

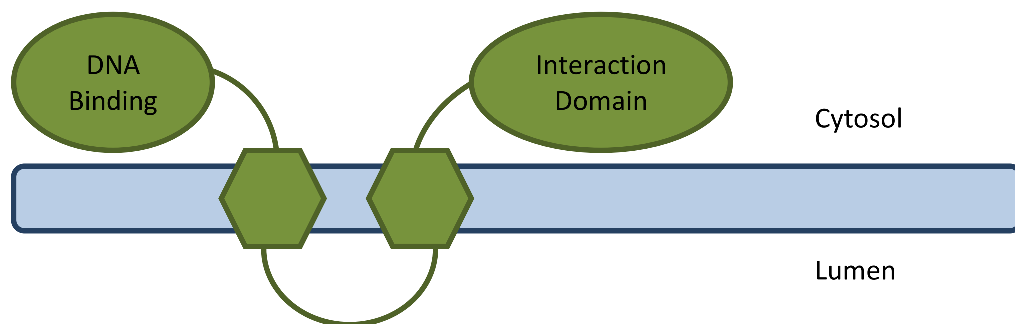

Figure 1: General structure of the SREBP transcription factors. The circle labeled DNA Binding is the basic helix-loop-helix-leucine zipper DNA binding domain. The hexagons are the membrane anchoring domain. The interaction domain is labeled as such.

The Transcriptional Regulation of SREBPs

The transcriptional regulation of SREBPs is controlled primarily by a few transcription factors, including SREBP itself. Both SREBP1c and SREBP2 have SREs sequences in their promoters (Chen 2004, Horton 2002). This allows SREBPs to drive their own expression, as well as each other's, in turn permitting SREBPs to alter their expression in response to cholesterol levels in the cell, as discussed below (Chen 2004, Horton 2002). Additionally the liver X receptor (LXR) can also modulate SREBP1c, as there are LXR elements found in the promoter of SREBP1c (Chen 2004, Dif 2006, Horton 2002, Jakobsson 2012, Xu 2013). The LXR drives expression of SREBP1c in response to signaling from the insulin receptor, increasing fatty acid synthesis (Chen 2004, Dif 2006, Horton 2002, Xu 2013). LxR activation from the insulin receptor is at the end of a long anabolic signaling cascade that involves mTOR among other proteins (Xu 2013). SREBP1a's promoter contains neither of these regulatory sequences, and so responds to neither of these signals (Fernandez 2008, Horton 2002).

Transport of SREBP from the ER to the Golgi

When SREBPs are first synthesized in the endoplasmic reticulum (ER) they associate with another membrane bound protein called SREBP cleavage-activating protein (SCAP) (Horton 2002, Nohturfft 2000, Peterson 2011, Sun 2007, Xu 2013, Yellaturu 2009). SCAP functions as a cholesterol sensor (Horton 2002, Nohturfft 2000,

Peterson 2011, Sun 2007, Xu 2013, Yellaturu 2009). When SCAP binds cholesterol it adopts a conformation that hides a sorting signal sequence (Sun 2007, Xu 2013). When this signal motif is hidden SCAP is able to bind to one of a pair of proteins called insulin-induced gene product 1 and 2a (Insig-1 and Insig-2a) (Yellaturu 2009). INSIG1 and 2a help keep SCAP and SREBP sequestered in the ER (Sun 2007, Xu 2013, Yellaturu 2009). As cholesterol concentrations go down SCAP changes conformation, revealing the sorting signal and disassociating from INSIG 1 and 2a (Sun 2007, Xu 2013, Yellaturu 2009). SCAP-SREBP is then transported to the Golgi in COPII vesicles (Sun 2007, Xu 2013, Yellaturu 2009). Once in the Golgi SREBP gets cleaved one time by both the site 1 protease (S1P) and site 2 protease (S2P), releasing SREBP's DNA binding domain (Hirano 2001, Horton 2002, Nohturfft 2000, Xu 2013). This process is also regulated through insulin signaling, at least partially through regulation of INSIG expression (Xu 2013, Yellaturu 2009). Insig-1 preferentially interacts with SCAP-SREBP2 while Insig-2a preferentially interacts with SCAP-SREBP1c (Yellaturu 2009). In response to insulin signaling Insig-2a expression goes down, releasing SCAP-SREBP1c to be transported to the Golgi regardless of the cholesterol concentration or other membrane lipid components (Yellaturu 2009).

Nuclear Localization of SREBP

Once released into the cytoplasm SREBPs then need to be transported to the nucleus and this process is also regulated by insulin signaling (Peterson 2011, Xu 2013). Signaling from the insulin receptor drives activity of mTOR which, among many other things, phosphorylates a protein called Lipin 1 (Peterson 2011, Xu 2013). When

phosphorylated Lipin 1 stays in the cytoplasm, but unphosphorylated Lipin 1 is able to move into the nucleus (Peterson 2011, Xu 2013) where it blocks the transport of SREBPs into the nucleus by methods that are currently poorly defined (Peterson 2011, Xu 2013). Once in the nucleus SREBP's half-life is regulated as well, and again insulin signaling plays a role (Xu 2013). In the nucleus SREBPs are known to be ubiquitinated by a cullin ubiquitin ligase and subsequently degraded by the proteasome (Hirano 2001, Xu 2013). In order to be ubiquitinated by a protein called F-box and WD repeat domain-containing 7 (Fbw7) SREBPs must first be phosphorylated (Xu 2013). Insulin signaling is able to inhibit this phosphorylation, thereby increasing SREBP's half-life and transcriptional activity (Xu 2013). A diagram of SREBP's transport throughout the cell is displayed in figure 2.

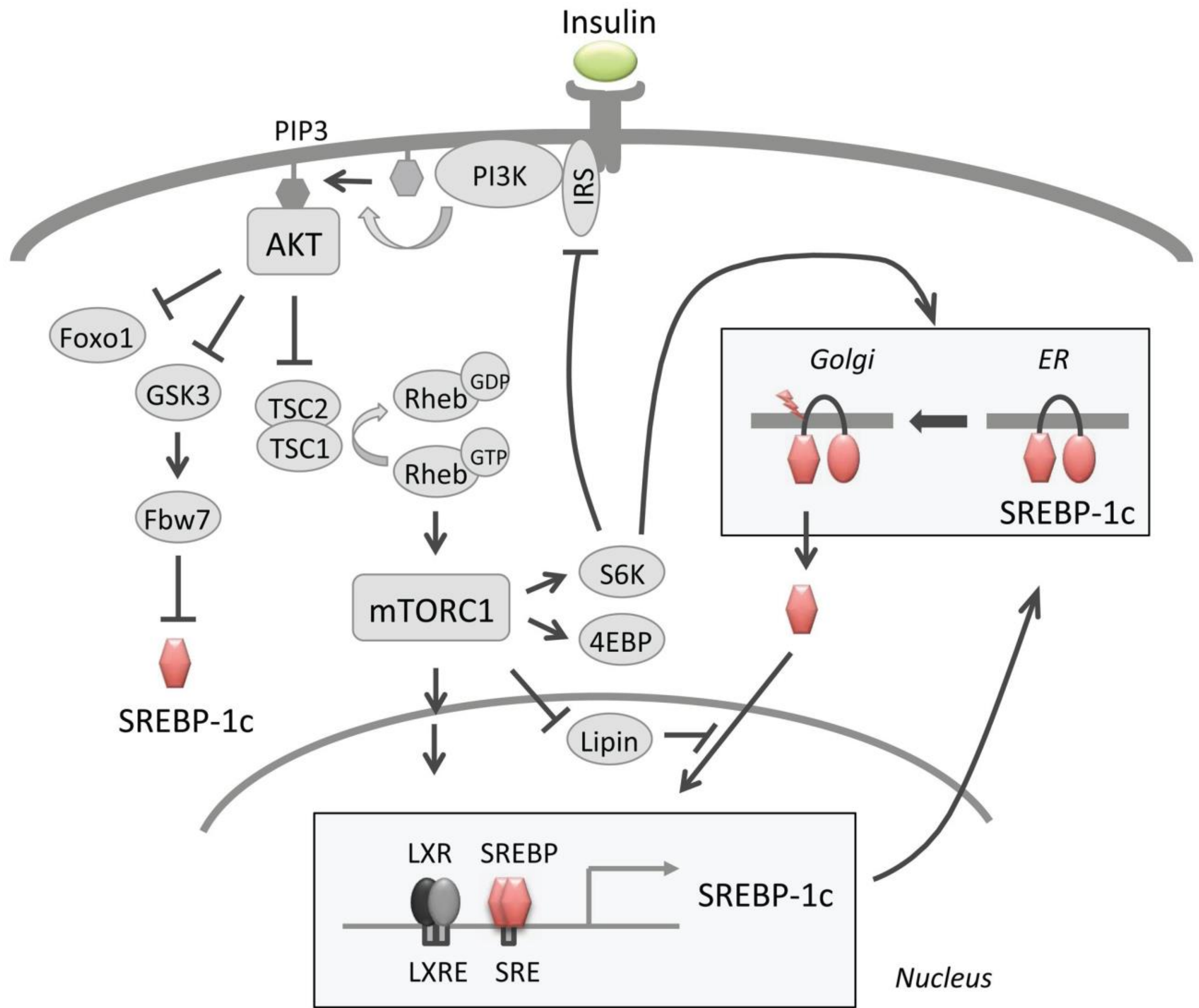

Figure 2: Diagram of the processes and signaling pathways involved in regulating SREBP1c. The other SREBPs lack the LxR element in their promoter, but are otherwise similar. The lightning bolt represents the cleavage event that releases the SREBPs DNA binding element from the Golgi membrane. Fbw7 is drawn in the cytoplasm, but its activity on SREBPs takes place in the nucleus (Xu 2013).

Design of This Thesis

The Takeda compound has some truly unique properties, but how the compound manifests its activity is not clear. Further development is somewhat hampered by not understanding the mechanism of action or the actual target of inhibition. The first logical step was to determine what aspects of SREBP activity are altered by the compound. A luciferase reporter assay was used to measure the effect of the compound on modulating expression from the various SREBP promoter sequences, independent of any effect on transport. Constitutive expression of chimeric gene fusions of GFP and SREBPs in liver cell lines was designed to track the progress of SREBPs through sub-compartments and organelles of the cell to characterize how the Takeda compound altered normal SREBP maturation and transport. Activity of the S1P and S2P proteases was observed by immunoblotting for full length and processed SREBP isoforms to see if the Takeda compound inhibited cleavage of the protein. Degradation of SREBP was measured by time laps imaging of the GFP tagged protein in the nucleus to see if the compound was able to increase degradation.

Chapter II

Materials and Methods

This section describes the experimental methods used in this thesis. Briefly plasmids containing both mutant and wild type SREBP promoters driving the expression of luciferase were created. Hek293 cells were transfected with these plasmids and their activities was measured using the dual luciferase assay. Plasmids containing GFP tagged SREBP were also created. These were used to transfect cells that were used in immunofluorescent assays to track SREBP transport.

Plasmid Construction

For the luciferase reporter plasmids 1 Kb of sequence directly upstream of the start codons of SREBP1A, SREBP1C, and SREBP2 were copied from the NCBI database (NCBI 2016). In addition to the wild type sequences two mutant sequences were designed where the two SRE sites in the SREBP1C promoter and the single SRE site in the SREBP2 promoter were scrambled to prevent self-regulation (Dif 2006, Sato 1996). SREBP1A does not contain an SRE site in its promoter so it was not necessary to create a mutant sequence (Horton 2002). The recognition sequence for the KpnI was added to the start of these five sequences and the recognition sequence for NheI was added to the end of them (NEB 2016 Kpn1, NEB 2016 Nhe1). These sequences were synthesized in pUC57-Kan plasmids by Genewiz. These five promoters were then cut out of the pUC57-

Kan plasmid and inserted into the pGL4.19 plasmid from Promega using T4 DNA ligase (NEB 2016 T4, Promega 2015 pGL4.19). The activity of these plasmids was tested using the luciferase assay described below.

To make the GFP plasmids the full length sequences for SREBP1A, SREBP1C, and SREBP2 were copied from existing plasmid constructs that had been synthesized using consensus WT cDNA sequence (Takeda N.D.). The sequence for GFP was added to the N terminus of each sequence as that is the portion of the protein that is transported to the nucleus. The three GFP-SREBP constructs were then synthesized and subcloned into pDEST40 plasmid (Invitrogen).

Plasmid Production

All of the luciferase reporter plasmids, the luciferase control plasmid, and the GFP plasmids were amplified in One-Shot® Top 10 chemically competent E. coli. E.coli were transformed according to the product insert (Invitrogen 2015). Cells were grown and plasmid was isolated according to the PureYealdTM Plasmid Midiprep Protocol (Promega 2016).

Cell Lines

HepG2, SNU-182, and Hek293 cells were obtained from ATCC. HepG2 cells were grown in MEM (Invitrogen) supplemented with 10% FBS (ATCC), 1% Non-Essential Amino Acids (Invitrogen), and 1% Sodium Pyruvate (Invitrogen). 2.5 mg/ml of

Geneticin (Invitrogen) was added to both select and grow resistant cell lines. SNU-182 cells were grown in RPMI (Invitrogen) supplemented with 10% FBS. 1.25 mg/ml was added to both select and grow resistant cells. Hek293 cells were grown in DMEM (Invitrogen) with 10% FBS. All cells were grown at 37°C and 5% CO_2.

Dual Luciferase Assay

The Dual-Glo® Luciferase Assay kit (Promega) was used to measure luciferase activity. 20,000 Hek293 cells were plated per well in a 96 well plate and were grown over night at 37°C and 5% CO2. Cells were then transiently transfected with both the luciferase reporter plasmid and the luciferase control plasmid at a 3:1 ratio using Lipofectamine 2000 according to its product insert (Invitrogen 2013). Cells were then treated overnight with 75 µl of experimental media (Promega 2015 Dual-Glo). Cells were then lysed in 75 µl of The Dual-Glo® Luciferase reagent per well and incubated for 10 min (Promega 2015 Dual-Glo). Firefly luciferase activity was then read in a PHERAstar FS (BMG LabTech). 75 µl of the Dual-Glow Stop & Glow reagent was then added to each well and incubated for 10 min (Promega 2015 Dual-Glo). Renilla luciferase activity was then read in the same PHERAstar FS.

Compound anti-proliferation EC_{50} determination

The ability of compounds to kill cells or inhibit cell growth was tested using the CellTiter-Glo® assay. For the two cell lines used in assays (SNU-182, Hek293) 1500 cells

were seeded per well in three 96 well plates. Cells were treated with 9 1:2 dilutions of drug compounds done in triplicate, starting at 10 μM for the Lipid, Uba6, Dual, mTOR, and S1P inhibitors and 2.5 μM for VELCADE. Cell viability was measured using the CellTiter-Glo® assay kit according to the manufacturer protocol (Promega 2015 Cell Titer-Glo). Survival curves were plotted and the EC50 was calculated for each drug in each cell line using standard curve fit algorithms (Takeda N.D.).

Testing Takeda Inhibitor's Effect on SREBP Expression

All five luciferase reporter plasmids (1AWT, 1CWT, 1CMUT, 2WT, and 2MUT) were tested using the dual luciferase assay. For each plasmid wells were treated with 5 different media (DMEM + 10% FBS, DMEM + 10% Lipoprotein deficient serum + 10% Lipoprotein concentrate, DMEM + 10% Lipoprotein deficient serum, DMEM, and DMEM + 100 nM Insulin) with 10 μM Lipid inhibitor, 10 μM Uba6 inhibitor, 5 μM Dual inhibitor, or 0.1% DMSO for a total of twenty conditions, all of which were made in sextuplets. Cells were incubated at 37°C and 5% CO_2 for 24 hours and then read according to the Dual Luciferase Assay. The 95% confidence intervals for the ratios of reporter luciferase and control luciferase were then calculated (Moore 2006).

Generating Stable N-GFP-SREBP Cell Lines

HepG2 and SNU-182 cells were seeded in a 6 well plate and transfected using Lipofectamine 2000 according to its product insert (Invitrogen 2013). After 3 days cells

were then switched to selective media (standard media + 2.5 mg/ml of Geneticin for HepG2 and 1.25 mg/ml of Geneticin for SNU-182). Media was exchanged every 5 days and the cultures were expanded once large colonies formed. Routine cell culture passaging in T75 flasks was implemented to keep cells in logarithmic growth.

Immunofluorescence

The antibodies used in immunofluorescence (IF) were tested with both HepG2 and SNU-182 cell lines. A rabbit anti-Calnexin antibody (Cell Signaling Technologies) was used to mark the ER and a mouse anti-GM130 antibody (BD Bioscience) was used to mark the Golgi. Secondary antibodies with fluorophores at wavelengths 594 and 647 (Invitrogen) were used. Cells were plated in a 96 well plate and fixed using methanol fixation (Takeda N.D.). The primary antibodies were used at 1:50 dilutions, the secondary antibodies were used at 1:600 dilutions, and DAPI was used at a 1:50,000 dilution. The plate was then read in a confocal fluorescent microscope using the DAPI, CY5, Texas Red, and FITC filters. All IF experiments were run in a similar manner.

Testing Takeda Inhibitor's Effect on SREBP Transport

Three T225 flasks of SNU-182 cells were transiently transfected with one of the three GFP tagged SREBP plasmids using Lipofectamine 2000 according to its product insert (Invitrogen 2013). These cells were incubated for 2 days before being harvested by trypsinization. They were then sorted for GFP expression using FACS according to

Takeda protocols into 8 wells of a 96 well plate (Takeda N.D.). The cells were then incubated for 12 hours, switched to treatment media and incubated for another 12 hours. They were then imaged by IF as described above.

Chapter III

Results

This section describes the results of the experiments. In summery the Takeda compounds are able to inhibit the activity of all promoters in growth conditions that do not contain serum. This suggests that they do not alter the transport of SREBP.

Compound anti-proliferation EC_{50} Results

The EC_{50} for the Uba6, Lipid, and Dual inhibitors used in the luciferase assay were measured in Hek293 cells using the CellTiter-Glo® assay and is recorded in Figure 3. The EC_{50} for the Dual inhibitor, S1P inhibitor, mTOR inhibitor, and Velcade were measured in SNU182 cells using the CellTiter-Glo® assay and is recorded in Figure 4.

The Uba6 and Lipid inhibitors showed no measurable toxicity even at the highest concentration tested so these compounds were used at a concentration of 10 μM in the luciferase assays. The Dual inhibitor is not particularly toxic, with significant toxicity only occurring at the 10 μM concentration and no concentration inhibited 50% of growth. For these cells a 5 μM concentration was chosen as a safe dose for use in experiments. This has also been shown to be an effective dose to observe the SREBP inhibition based on previous research at Takeda (Takeda N.D.).

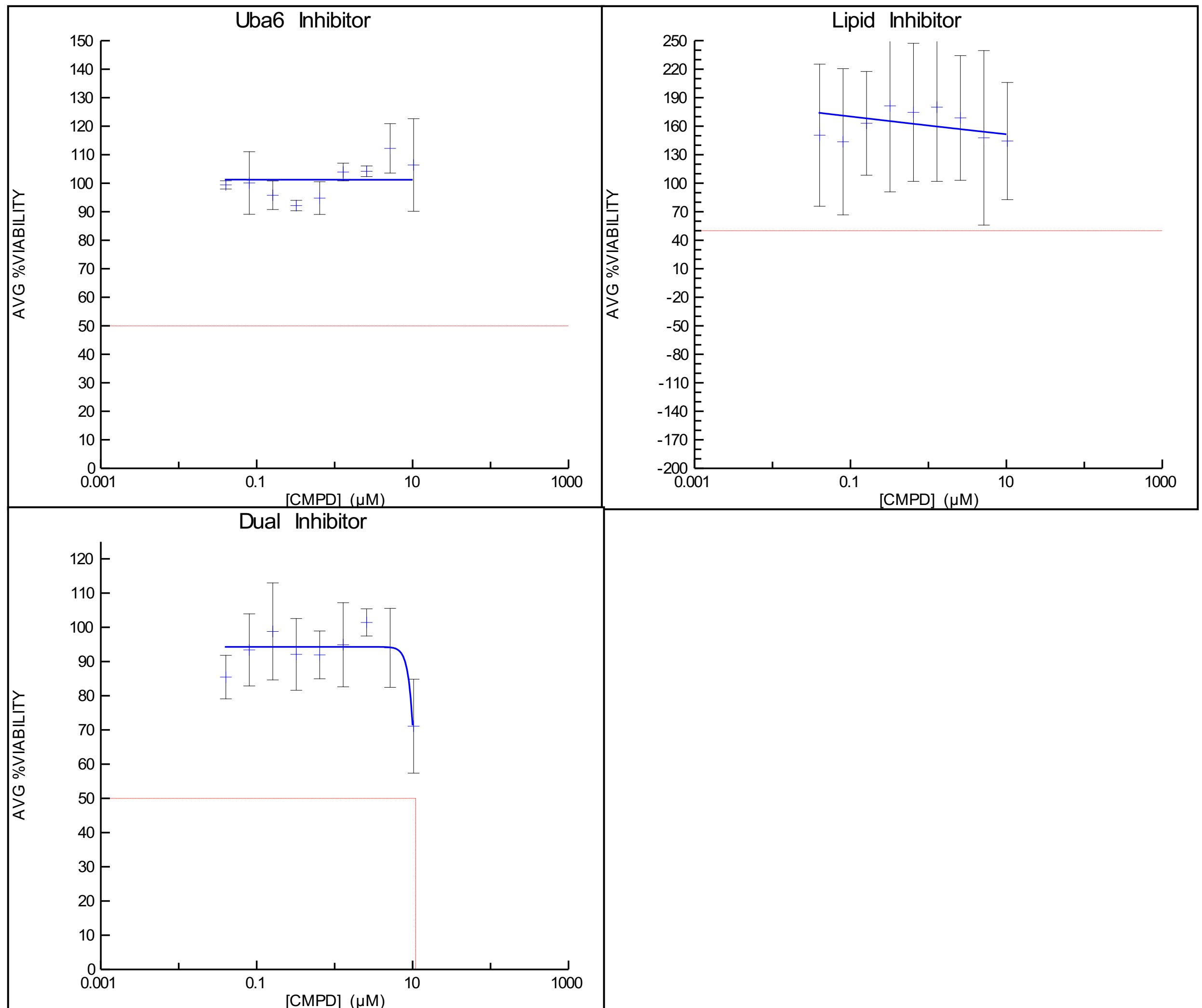

Figure 3: Effects of the inhibitors on Hek293 cell's viability. Blue + signs are average values calculated from the three replicates. The blue line is a line fit to the observed effect of the change in compound concentration on cell viability. The red dashed line is the predicted EC50. Error bars are the black vertical lines.

The SNU182 cells used in the IF assays were tested with the Dual, S1P, and mTOR inhibitors, as well as Velcade. The results are listed in Figure 4. Again the Dual inhibitor is not very toxic, never reaching 50% inhibition at any concentration. 5 μM was again chosen as an acceptable dose for experiments. The S1P inhibitor had a calculated EC50 of 4.75 μM and a concentration of 1 μM was chosen for use in further experiments. The mTOR inhibitor had a calculated EC50 of 0.30 μM and 1 μM was chosen as the working concentration for it. While this was higher than the EC50 concentration this test exposed the cells to compound for 3 days. In the IF experiments cells were only going to be exposed to compound for 24 hours, which should produce less inhibition. VELCADE killed almost 90% of cells at all concentrations tested, making it impossible to calculate an EC50. The individual reads for VELCADE treatments were also much more consistent than for the other compounds, resulting in small or apparently missing error bars. Past research has shown VELCADE to be toxic at very low concentrations in many cell lines so this result is not unexpected (Adams 1999). VELCADE is highly toxic to cells in culture at higher concentrations, but at lower concentrations induction of apoptosis and cell death does not occur significantly until after 24 hours, so a 0.1 μM concentration was chosen for use in experiments (Takeda N.D.).

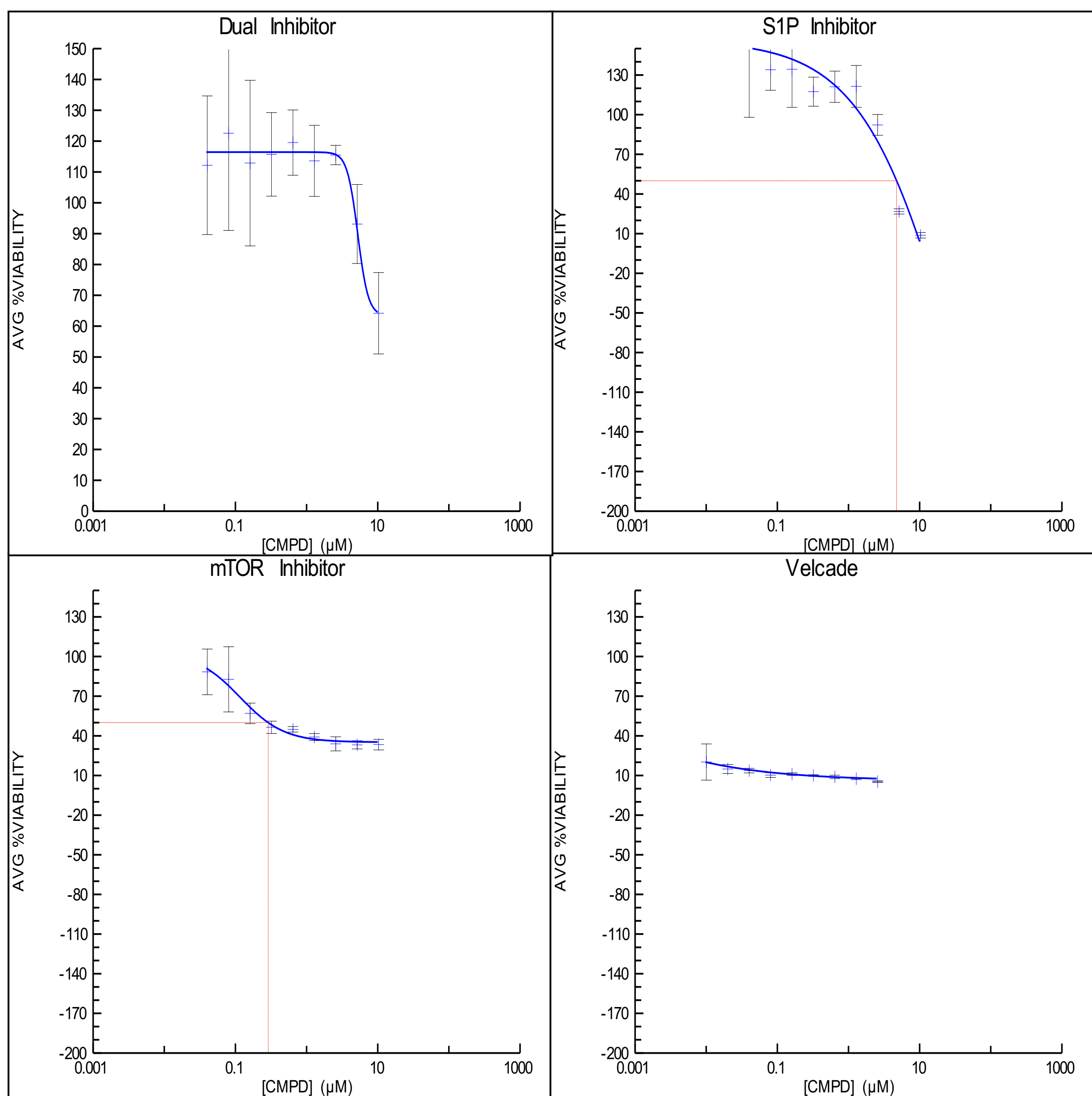

Figure 4: Effects of the 4 compounds on SNU182 cell's viability. Blue + signs are average values calculated from the three replicate plates. The blue line is a line fit to the observed effect of the change in compound concentration on cell viability. The red dashed line is the calculated EC50. Error bars are the black vertical lines.

Takeda Compounds' Effect on SREBP Expression

The dual luciferase assay uses two separate plasmids, a reporter plasmid and a control plasmid. The control plasmid contains the Renilla luciferase transcribed by a constitutive CMV promoter (Takeda N.D). The second contains the firefly luciferase driven by the inserted experimental promoters derived from the 1 kb upstream region of the various SREBP genes. The luminescence signals of the two promoters are measured and compared within a single sample. By using the ratio of firefly luciferase activity from the reporter plasmid relative to the Renilla luciferase control plasmid construct it is possible to compare promoter activity while controlling for differences in transfection efficiency, general transcriptional effects, and cell viability (Alam 1990, Promega 2015 Dual-Glo). In this experiment 5 different reporter plasmids were used: SREBP1AWT, SREBP1CWT, SREBP1CMUT, SREBP2WT, and SREBP2MUT. HEK293 cells were transfected with these plasmids, and grown in the 5 different media treated with either the Lipid inhibitor, Uba6 inhibitor, Dual inhibitor, or 0.1% DMSO. The +/- INS media is DMEM with or without 100nM insulin. These growth conditions allow the effect of insulin signaling to be measured. The +/- CHO media is DMEM + 10% lipoprotein deficient serum with or without 10% cholesterol concentrate. These growth conditions allow the effect of cholesterol depravation to be measured. +FBS is DMEM + 10% FBS which is the standard growth media for these cells. The 95% confidence intervals for the luciferase ratios were calculated and are recorded in Figures 5, 6, 7, and 8.

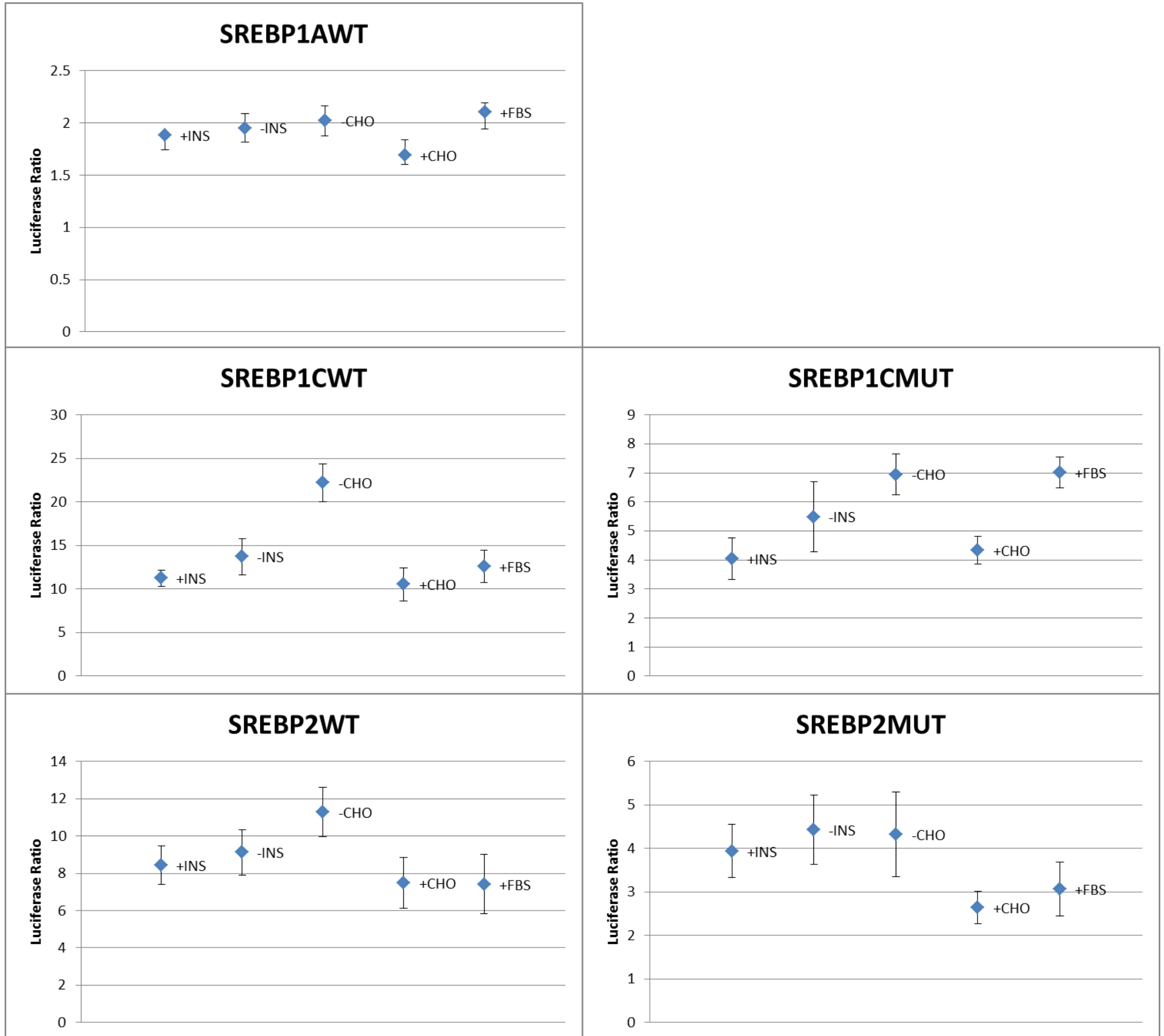

Figure 5: 95% confidence intervals for luciferase ratios of DMSO treated cells. Each plot has the values for one reporter plasmid, sorted by growth condition. Ratios are calculated by dividing the luminescence of the reporter plasmid by the luminescence of the control plasmid.

The SREBP1a promoter is known to not increase expression in response to insulin or cholesterol depravation. In figure 5 SREBP1A has a small but significant increase in expression. Wild type SREBP1A (SREBP1AWT) has no SRE element and therefore should be unable to respond to changes in cholesterol concentration. This effect also appears with the two mutant promoters (SREBP1CMUT, SREBP2MUT) that have had their SRE elements removed, where a smaller response could be detected relative to the WT reporters. The media conditions for testing the effects of cholesterol as well as the locations of the SRE elements were taken from peer reviewed literature so it is unclear why the expected results were unable to be replicated. While these results were unexpected they could potentially be explained by incomplete deletion of the SRE element or an alternative regulation pathway.

The SREBP1c promoters were expected to increase expression in response to insulin but this was not observed. Immortalized cell lines have been reported to not respond to insulin signaling the way normal cells would, and this may explain the observed result. The SREBP1A and SREBP2 promoters did not respond to insulin signaling as was expected. It is also worth noting that the two mutant promoters had lower expression ratios than their wild type counterparts, which is the expected result of removing the SRE element.

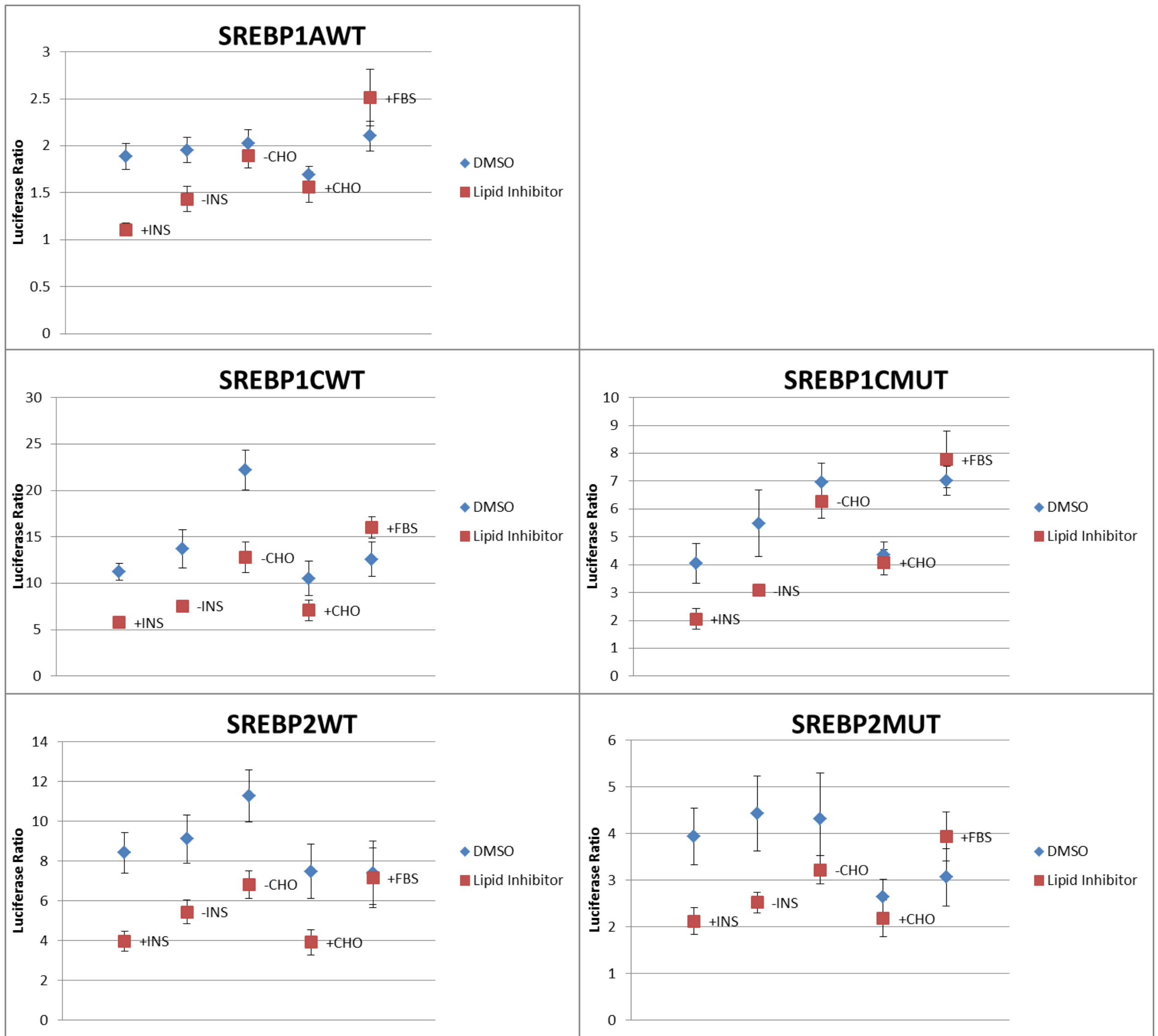

Figure 6: 95% confidence intervals for luciferase ratios of Lipid inhibitor treated cells. Each plot has the values for one reporter plasmid complaining Lipid inhibitor treated cells (Red) with DMSO treated cells (Blue), sorted by growth condition. Ratios are calculated by dividing the luminescence of the reporter plasmid by the luminescence of the control plasmid.

As seen in figure 6 the Lipid inhibitor is able to reduce the expression of all the reporter plasmids in growth conditions that don't include serum of some kind. In the two wild type promoters it is also able to inhibit expression in the presence of lipoprotein deficient serum. The magnitude of this effect doesn't seem to change based on the presence or absence of cholesterol and insulin suggesting it targets some other regulatory mechanism. The fact that the Lipid inhibitor works with all the plasmids regardless of whether they have a SRE element or not also suggests that the observed effect is not from inhibiting SREBP transport, but directly at the level of transcription. As the dual luciferase assay controls for cell viability and drug doses were chosen to have limited proliferative effects, it is unlikely that these results are due to general toxicity of the compounds instead of their specific effects.

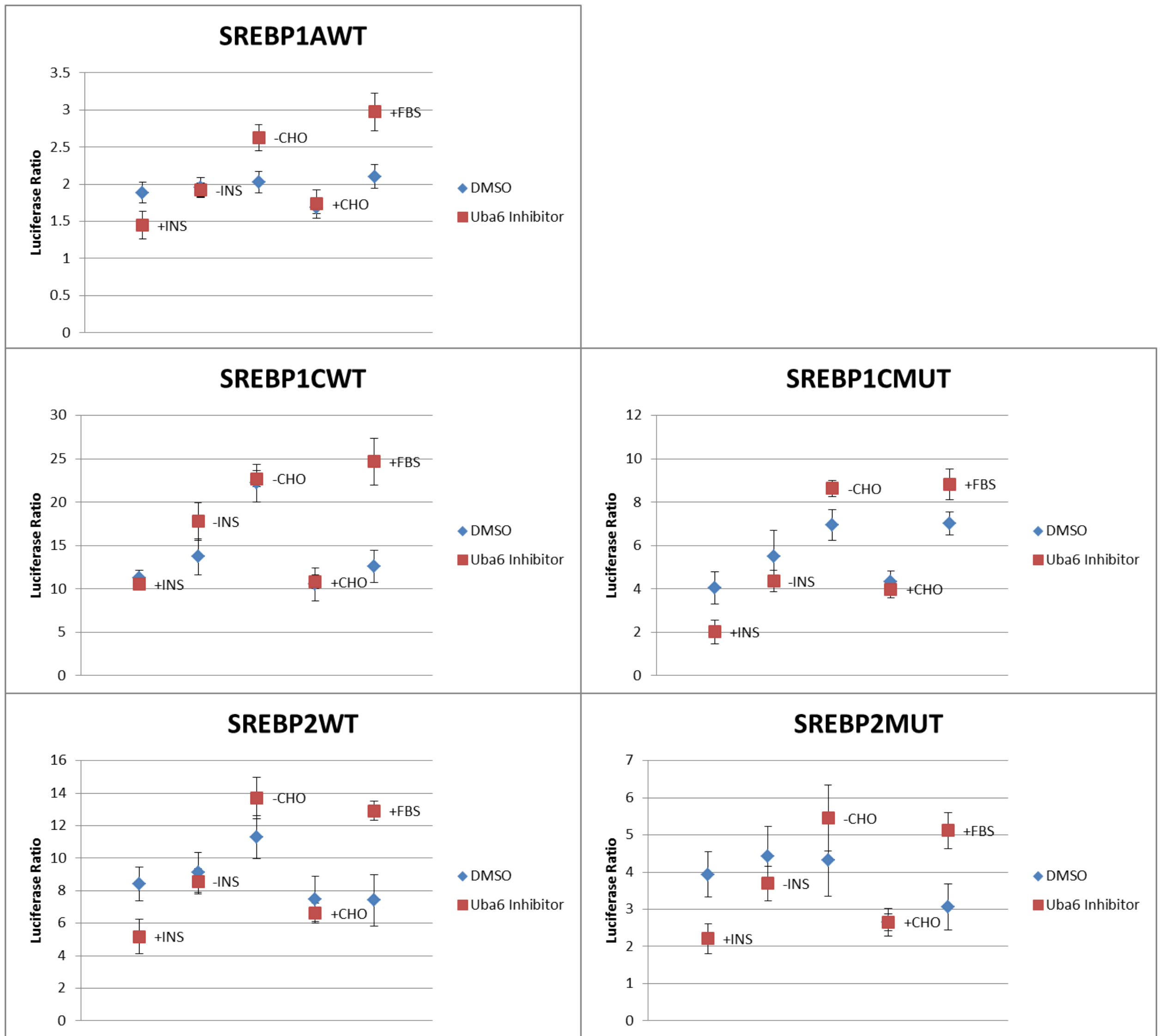

Figure 7: 95% confidence intervals for luciferase ratios of Uba6 inhibitor treated cells. Each plot has the values for one reporter plasmid complaining Uba6 inhibitor treated cells (Red) with DMSO treated cells (Blue), sorted by growth condition. Ratios are calculated by dividing the luminescence of the reporter plasmid by the luminescence of the control plasmid.

Uba6 is not known to regulate lipid or cholesterol synthesis so it was not expected to regulate the expression of SREBP. Figure 7 does show that the Uba6 inhibitor appears to inhibit SREBP expression in +INS media and promotes expression in +FBS media with all promoters. For the most part in other conditions it has no effect.

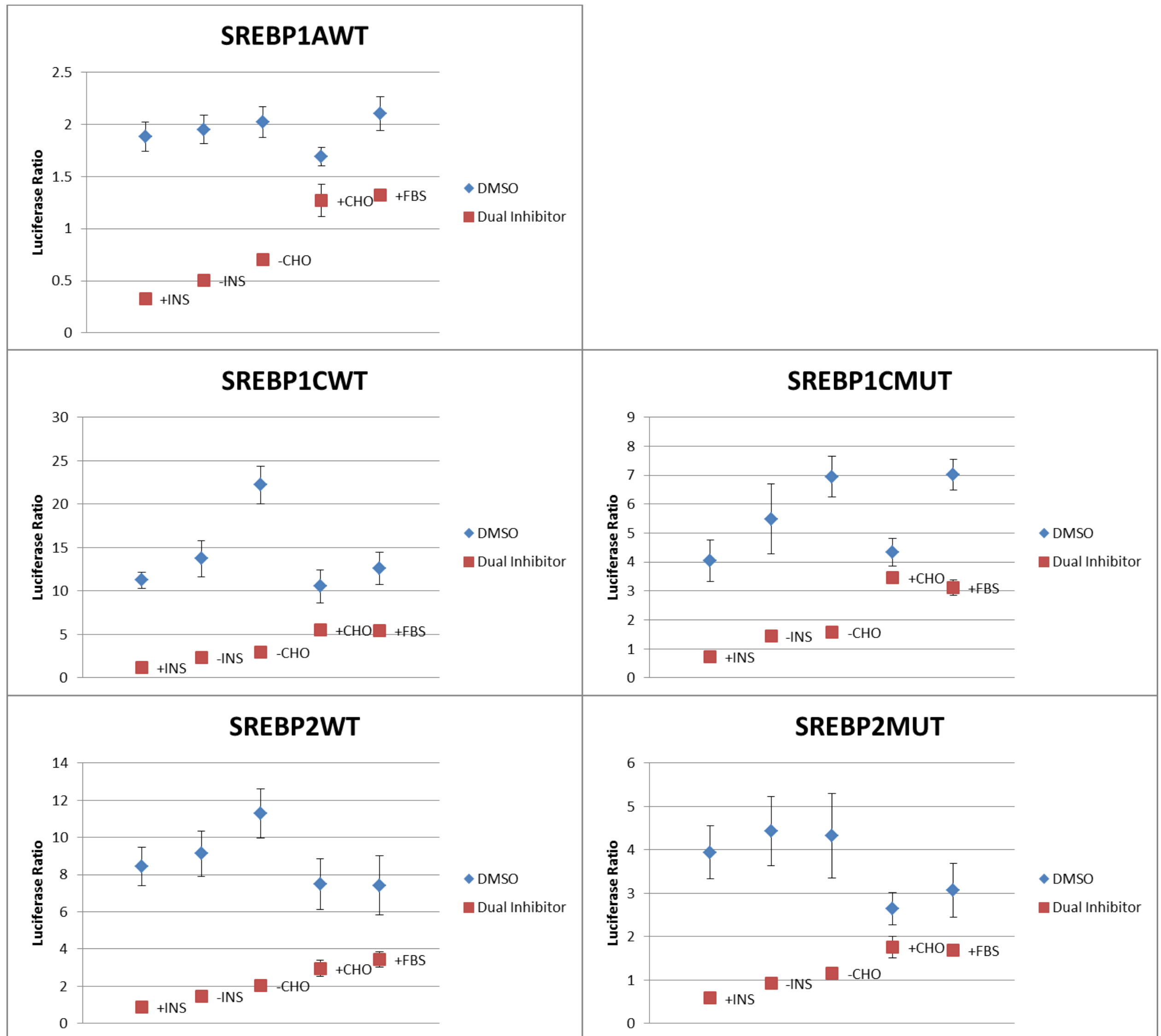

Figure 8: 95% confidence intervals for luciferase ratios of Dual inhibitor treated cells. Each plot has the values for one reporter plasmid complaining Dual inhibitor treated cells (Red) with DMSO treated cells (Blue), sorted by growth condition. Ratios are calculated by dividing the luminescence of the reporter plasmid by the luminescence of the control plasmid.

The Dual inhibitor is able to inhibit both Uba6 and cause the SREBP target gene expression. Figure 8 shows that it is able to inhibit all the promoters in all media. The effect is largest in media without cholesterol (+/- INS, -CHO) and does not seem to change depending on the presence or absence of an SRE element in the promoter. It also does not seem to be influenced by insulin signaling. Again this suggests that the Dual inhibitor exerts its effects through inhibiting SREBP expression and not its transport.

Chapter IV

Discussion

The fact that SREBP1C and SREBP2 regulate their own expression makes it more challenging to define how a compound alters their transcriptional activity. A compound that inhibits SREBP transport or processing would also appear to alter its expression. A compound that inhibits just one of the SREBPs could possibly also indirectly affect others as well through this regulation. Comparing the wild-type promoters to mutant promoters with scrambled SRE elements makes it possible to isolate where the inhibitory effect is coming from. A compound that inhibits the transport or processing of SREBP would be predicted to have no differential effect on the mutant promoters relative to the wildtype.

The compounds used in the dual luciferase assay were selected to help isolate the observed effect on SREBP expression. The Uba6 and Dual inhibitors are able to inhibit Uba6 at roughly the same concentration while the Lipid inhibitor was unable to do so at any tested concentration [Gavin 2011, Takeda N.D.]. Conversely the Lipid and Dual inhibitors are both known to inhibit expression of SREBP target genes while the Uba6 inhibitor is not [Takeda N.D.]. The observed activity that is shared between the lipid and dual inhibitors would be expected to be responsible for the SREBP target inhibition observed in the original tests. The Dual inhibitor had been shown in earlier experiments to be better at inducing this effect than the Lipid inhibitor [Takeda N.D.]. This result was recreated in these

experiments with the Dual inhibitor exhibiting a much stronger effect even though it is being used at half the concentration of the Lipid inhibitor.

The expression of various SREBPs are regulated by a complex set of signals that respond to anabolic stimulus that include insulin and cholesterol levels. Looking at which reporter plasmids are inhibited by the Takeda compound would be predicted to inform how the compound achieves its repressive effects on SREBP dependent transcription. SREBP1C is the only SREBP promoter able to respond to insulin signaling, so a compound that only affects those promoters would be expected to inhibit insulin signaling. If only the SREBP1CWT and SREBP2WT promoters are affected that would suggest SREBP transport is being inhibited somehow. SREBP1A responds to neither insulin nor cholesterol levels, so a compound that inhibits it must exert its affect though some means that doesn't involve either signal.

As was shown in these experiments the Lipid and Dual inhibitors inhibit expression in all of the reporter plasmids. The relative magnitude of the inhibition does not seem to be affected by the presence or absence of SRE elements in the promoters though cholesterol and/or serum seem to be involved. The most likely possibility is that these compounds inhibit some transcription factor or enhancer element, either directly or indirectly, that is shared by all three SREBP gene promoters rather than inhibit the transport of SREBP through the cell. The promoters of all three SREBPs are known to contain binding sites for Sp1 and NF-Y but there could be other unknown shared transcription factors (Chen 2004, Fernandez 2008, Sato 1996). The 1 Kb promoter sequences used in this thesis are significantly longer than previously described promoter sequences (Chen 2004, Fernandez 2008, Sato 1996). Therefore it is also possible that

there are additional unreported SRE sequences in each promoter region, but this seems unlikely especially for SREBP1A which is not reported to respond to cellular cholesterol levels.

The original intent of this thesis had been to investigate the transport of GFP tagged SREBP throughout the cell. Numerous problems were encountered and the assay was never successful. The plasmids themselves seem to be toxic to cells, killing most of the HepG2 and SNU182 cells that were transfected within a few days. Those that remained expressed visible amounts of GFP and were resistant to Geneticin selection though they didn't grow. After about a month the remaining cells died, leaving behind a single colony of growing cells. While early on GFP was expressed in the ER these resistant cells expressed it in specks that lightly co-stained with the Golgi marker. HepG2 cells were very clumpy, round, and poorly suited to IF work so the SNU182 cells were used. In these cells none of the control compounds altered the localization of GFP tagged SREBP. In a second attempt transiently transfected cells were sorted by FACS to get sufficient numbers for IF. Again none of the control compounds altered GFP-SREBP localization. Representative image of these cells are shown in figure 9. This problem with the transport of GFP tagged SREBP made attempts to measure cleavage by S1P and nuclear cleavage unsuccessful as well. Using a different tag or screening other cell lines may be a potential solution to this obstacle.

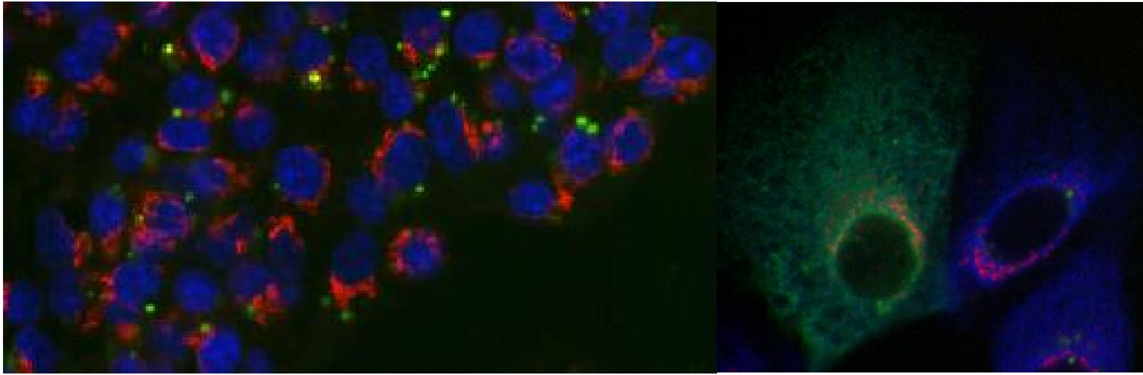

Figure 9: Representative images from IF experiments.
Left HepG2 cells stably expressing N-GFP-SREBP. Blue: Nucleus, Red: Golgi, Green: GFP.
Right SNU182 cells transiently expressing N-GFP-SREBP. Blue: ER, Red: Golgi, Green: GFP.

This thesis has provided data about the general mechanism of action of the Takeda compounds and has provided the basis for additional avenues of research that can be taken to further investigate them. One possibility would be to identify regulatory elements shared amongst all SREBPs that when removed would prevent the drug from having its effect. A screening approach could be attempted by systematically removing or testing smaller elements of each promoter. Additionally a compound focused approach could be attempted by modifying the compounds with an activatable crosslinker and look for what proteins interact with the compounds thereby defining candidate molecular targets. The effects of these compounds are intriguing and this research may contribute to the development of a novel treatment for NASH, a disease with a significant and growing health burden.

References

Adams J., Palombella V.J., Sausville E.A., Johnson J., Destree A., Lazarus D.D., Maas J, Pien C.S., Prakash S., Elliott P.J. (1999) Proteasome Inhibitors: A Novel Class of Potent and Effective Antitumor Agents. *Cancer Research* 59:2615-2622

Alam J., Cook J.L. (1990) Reporter Genes: Application to the Study of Mammalian Gene Transcription. *Analytical Biochemistry* 188: 245-254

Amidon B.S., Cardin D.P., Gould A.E., Greenspan P.D., Harrison S.J. (2014) Indole-Substituted Pyrrolopyrimidinyl Inhibitors of Uba6. https://patentimages.storage.googleapis.com/pdfs/58db81781b7bf696c37b/US20150210700A1.pdf. Accessed March 1, 2017

Angulo P. (2002) Nonalcoholic Fatty Liver Disease. *New England Journal of Medicine*. 346(16): 1221-1231

Chen G., Liang G., Ou J., Goldstein J.L., Brown M.S. (2004) Central Role for Liver X Receptor in Insulin-Mediated Activation of SREBP-1c Transcription and Stimulation of Fatty Acid Synthesis in Liver. *Proceedings of the National Academy of Sciences of the United States of America.* 100(31): 11245-11250

Dif N., Euthine V., Gonnet E., Laville M., Vidal H., Lefai E. (2006) Insulin Activates Human Sterol-Regulatory-Element-Binding Protein-1c (SREBP-1c) Promoter Though SRE Motifs. *The Biochemical Journal* 400: 179-188

Fernández-Álvarez A., Tur G., López-Rodas G., Casado M. (2008) Reciprocal Regulation of the Human Sterol Regulatory Element Binding Protein (SREBP)-1a Promoter by Sp1 and EGR-1 Transcription Factors. *Federation of European Biochemical Societies Letters*. 582: 177-184

Gavin J.M., Chen J.J., Liao H., Rollins N., Yang X., Xu Q., Ma J., Loke H., Lingaraj T., Brownell J.E., Mallender W.D., Gould A.E., Amidon B.S., Dick L.R. (2011) Mechanistic Studies on Activation of Ubiquitin and Di-ubiquitin-like Protein, FAT10, by Ubiquitin-like Modifier Activating Enzyme 6, Uba6. *The Journal of Biological Chemistry* 287(19): 15512-15522

Hirano Y., Yoshida M., Shimizu M., Sato R. (2001) Direct Demonstration of Rapid Degradation of Nuclear Sterol Regulatory Element-Binding Proteins by the Ubiquitin-Proteasome Pathway. *The Journal of Biological Chemistry.* 279(39): 36431-36437

Horton J.D., Goldstein J.L., Brown M.S. (2002) SREBPs: Activators of the Complete Program of Cholesterol and Fatty Acid Synthesis in the Liver. *The Journal of Clinical Investigation* 109(9): 1125-1131

Invitrogen (2013) Lipofectamine 2000 Product Insert. https://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_2000_Reag_protocol.pdf . Accessed October 22, 2016

Invitrogen (2015) One-Shot Top10 Chemically Competent E. coli Product Information Sheet. https://tools.thermofisher.com/content/sfs/manuals/oneshottop10_chemcomp_man.pdf Accessed July 14, 2016

Jakobsson T., Treuter E., Gustafsson J.Å., Steffensen K.R. (2012) Liver X Receptor Biology and Pharmacology: New Pathways, Challenges and Opportunities. *Trends in Pharmacological Sciences.* 33(7): 394-404

Machado M.V., Cortez-Pinto H. (2014) Non-Alcoholic Fatty Liver Disease: What the Clinician Needs to Know. *World Journal of Gastroenterology.* 20(36): 12956-12980

Moore D.S., McCabe G.P. (2006) Introduction to the Practice of Statistics: Fifth Edition. New York: W.H. Freeman and Company

National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov Accessed July 12, 2016

New England BioLabs (NEB) KpnI Product Insert. https://www.neb.com/products/r0142-kpni. Accessed July 12, 2016

New England BioLabs (NEB) Nhe1 Product Insert. https://www.neb.com/products/r0131-nhei. Accessed July 12, 2016

New England BioLabs (NEB). T4 DNA Ligase Protocol https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202. Accessed September 24, 2016

Nohturfft A., Yabe D., Goldstein J.L., Brown M.S, Espenshade PJ. (2000) Regulated Step in Cholesterol Feedback Localized to Budding of SCAP from ER Membranes. *Cell.* 102: 315-323

Peterson T.R., Sengupta S.S., Harris T.E., Carmack A.E., Kang S.A., Balderas E., Guertin D.A., Madden K.L., Carpenter A.E., Finck B.N., Sabatini D.M. (2011) mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. *Cell.* 146: 408-420

Promega (2015) CellTiter-Glo Luminescent Cell Viability Assay Technical Bulletin. https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol.pdf. Accessed October 6, 2016

Promega (2015) Dual-Glo Luciferase Assay System Technical Manual. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/dual-glo-luciferase-assay-system-protocol.pdf . Accessed January 20, 2017

Promega (2015) pGL4.19[luc2CP/Neo] Vector Product Information. http://www.promega.com/resources/protocols/product-information-sheets/a/pgl419-vector-protocol . Accessed July 12, 2016

Promega (2016) PureYeald Plasmid Midiprep System Technical Manual. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/pureyield-plasmid-midiprep-system-protocol.pdf. Accessed July 26, 2016

Ratziu V., Goodman Z., Sanyal A. (2015) Current Efforts and Trends in the Treatment of NASH. *Journal of Hepatology.* 62:S65-S75

Sato R., Inoue J., Kawabe Y., Kodama T., Takano T., Maeda M. (1996) Sterol-Dependent Transcriptional Regulation of Sterol Regulatory Element-Binding Protein-2. *The Journal of Biological Chemistry*. 271(43): 26461-26464

Sun L., Seemann J., Goldstein J.L., Brown M.S. (2007) Sterol-Regulated Transport of SREBPs from Endoplasmic Reticulum to Golgi: Insig Renders Sorting Signal in Scap Inaccessible to COPII Proteins. *Proceedings of the National Academy of Sciences of the United States of America.* 104(16): 6519-6526

Takeda (N.D.) Unpublished Internal Takeda documents

Vernon G., Baranova A., Younossi Z.M. (2011) Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Alimentary Pharmacology and Therapeutics* 34: 274-285

Xu X., So J., Park J., Lee A. (2013) Transcriptional Control of Hepatic Lipid Metabolism by SREBP and ChREBP. *Seminars in Liver Disease.* 33(4): 301 – 311

Yellaturu C.R., Deng X., Park E.A., Raghow R., Elam M.B. (2009) Insulin Enhances the Biogenesis of Nuclear Sterol Regulatory Element-Binding Protein (SREBP)-1c by Posttranscriptional Down-Regulation of Insig-2A and Its Dissociation from SREBP Cleavage-Activating Protein (SCAP)-SREBP-1c Complex. *The Journal of Biological Chemistry.* 284(46): 31726-31734