Discovery and Characterization of a Novel Fatty Acid Synthase Inhibitor with Antineoplastic Activity Against Breast Cancer

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Neil Spector

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

During oncogenesis, cancer cells go through metabolic reprogramming to maintain their high growth rates and adapt to changes in the microenvironment and the lack of essential nutrients. Several types of cancer are dependent on de novo fatty acid synthesis to sustain their growth rates by providing precursors to construct membranes and produce vital signaling lipids. Fatty acid synthase (FASN) catalyzes the terminal step of de novo fatty acid synthesis and it is highly expressed in many types of cancers where it’s up-regulation is correlated with cancer aggressiveness and poor therapeutic outcome. Several FASN inhibitors were developed and showed potent anticancer activity. However, only one inhibitor advanced to early stage clinical trials with some dose limiting toxicities. Using a modified fluorescence-linked enzyme chemoproteomic strategy (FLECS), we identified HS-106, a thiophenopyrimidine FASN inhibitor that displays anti-neoplastic activity against breast cancer in vitro and in vivo. HS-106 was able to inhibit purified human FASN and cellular fatty acid synthesis activity as evaluated by radioactive tracers’ incorporation into lipids. In proliferation and apoptosis assays, HS-106 was able to block proliferation and induce apoptosis in several breast cancer cell lines. Several rescue experiment and global lipidome analysis were performed to probe the mechanism by which HS-106 induces apoptosis. HS-106 was found to induce several changes in lipids metabolism including: (i) inhibition of fatty
acids synthesis. (ii) Inhibition of fatty acids oxidation as suggested by the blocking of HS-106 induced apoptosis through the inhibition of malonyl-CoA accumulation and the increase in the abundance of ceramides. (iii) Increase fatty acids uptake and neutral lipids formation as confirmed by $^{14}$C Palmitate uptake assay and neutral lipids staining. (iv) Inhibition of phospholipids formation by inhibiting de novo fatty acid synthesis and diverting exogenous fatty acids into neutral lipids. All of these events would lead to disruption in cellular membranes structure and function. HS-106 activity was also evaluated in Lapatinib resistant cell lines and it was able to induce apoptosis and synergizes Lapatinib activity in these cell lines. This may be due to the disruption of lipid rafts based on the observation that HS-106 reduces the expression of both HER2 and HER3. HS-106 was found to be well tolerated and bioavailable in mice with high elimination rate. HS-106 efficacy was tested in MMTV neu mouse model. Although HS-106 did not significantly reduced tumor size, it was able to double the median survival of the mice and show potent antitumor activity when combined with Carboplatin. Similar results were obtained when same combinations and dosing schedule was used in the triple negative C3Tag mouse model except for the inability of HS-106 to affect mice survival.

Taken together, HS-106 represents a novel FASN inhibitor that has anticancer activity both in vivo and in vitro. Being a chemically tractable molecule, the synthetic route to HS-106 is readily adaptable for the synthesis of analogs with enhanced activity.
Dedication

To my parents, for their unconditional love and support.
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List of Abbreviations

AAF-AMC: Alanine-Alanine-Phenylalanine-7-Amino-4-methylcoumarin

ACC: Acetyl-CoA carboxylase

ACLY: ATP-citrate lyase

ACN: Acetonitrile

ACP: Acyl carrier protein

ACS: Acetyl-CoA synthetase

ADP: Adenosine Diphosphate

AMC: 7-Amino-4-methylcoumarin

AMPK: 5' AMP-activated protein kinase

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

bHLH-Zip: basic helix-loop-helix leucine zipper transcription factors

Biw: Biweekly dosing

BSA: Bovine serum albumin

CBC: Complete blood count

CPT-1: Carnitine Palmitoyl transferase 1

DAPI: 4', 6-diamidino-2-phenylindole

DAPK: Death-associated protein kinase 3

DEVD: Aspartate-Glutamine-Valine-Aspartate
DH: Dehydrase

DMEM: Dulbecco’s Modified Eagle Medium

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EGCG: Epigallocatechin gallate

EGF: Epidermal Growth Factor

EIC: Extracted ion chromatograms

ER: Enoyl reductase

ESI: Electrospray ionization

FASN: Fatty acid synthase

FBS: Fetal Bovine Serum

FLECS: fluorescence linked enzyme chemoproteomic strategy

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GF-AMC: Glutamine-Phenylalanine-7-Amino-4-methylcoumarin

Hct: Hematocrit

Hgb: Hemoglobin

HSF-1: Heat shock factor protein 1

Hsp70: Heat Shock Protein 70
Hsp90: Heat Shock Protein 90

HT: High-Throughput

i.p.: Intraperitoneal

IPA: Isopropyl alcohol

IRAK1: interleukin-1 receptor-associated kinase 1

KAS: Ketoacyl synthase

KR: Ketoacyl reductase

KRAS: Kirsten rat sarcoma viral oncogene homolog

LCMS: Liquid chromatography–mass spectrometry

LYMF: lymphocytes

MALDI-MS: matrix-assisted laser desorption-ionization mass spectrometry

MAT: Malonyl/acetyl transferase

MCD: Malonyl-CoA decarboxylase

MMTV: Mouse Mammary Tumor Virus

MP1U: Mouse Phase 1 Unit

MS: mass spectrometry

MTBE: Methyl tert-butyl ether

MTD: Maximum Tolerated Dose

mTOR: mammalian target of rapamycin

NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NS5: Dengue virus nonstructural protein 5
PBS: phosphate-buffered saline
PBS-T: PBS with 0.01% Tween-20
PFA: Paraformaldehyde
PfPK9: Plasmodium falciparum protein kinase 9
PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PK: Pharmacokinetics
PVDF: polyvinylidene difluoride
Qd: daily dosing
RBC: red blood cell count
RFU: relative fluorescence units
Rh110: Rhodamine 110
SAR: structure-activity relationship
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC7A5: Solute carrier family 7 member 5
SREBP: Sterol regulatory element-binding protein
TBS: Tris-buffered saline
TBS-T: TBS with 0.01% Tween-20
TCA: Tricarboxylic acid cycle
TE: Thioesterase

TIC: total ion chromatogram

TLC: thin-layer chromatography

TOF/TOF: time of flight/ time of flight mass spectrometry

TRAP-1: TNF receptor-associated protein 1

UPLC: Ultra High Performance Liquid Chromatography

w.w.: wet weight

WBC: White blood cell count


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1. Introduction

1.1 De novo fatty acid synthesis

Living cells require fatty acids as precursors to construct membranes and produce vital signaling lipids (Brusselmans and Swinnen, 2009). In addition to being important energy storage molecules, fatty acids are needed for certain protein post translational modifications that are essential for maintaining and regulating protein functions (Iwanaga et al., 2009). In mammals, fatty acids are synthesized through the de novo fatty acid synthesis pathway in a limited number of tissues, while most of tissues obtain their need of fatty acids from the circulation (Swinnen et al., 2006). The main product of de novo fatty acid synthesis is the 16 carbon acyl-chain palmitate, which is synthesized by priming a condensation reaction with acetyl-CoA and multiple cycles of malonyl-CoA addition to the acyl-chain in the presence of NADPH which is used to saturate the growing acyl-chain (Smith and Abraham, 1970).

Precursors for fatty acid synthesis are provided by different metabolic pathways; acetyl-CoA is provided from Acetate by Acetyl-CoA synthetase (ACS) or Citrate from TCA cycle by ATP-Citrate lyase (ALCY). Acetyl-CoA can also be carboxylated by acetyl-CoA Carboxylase (ACC) to provide malonyl-CoA and the pentose phosphate pathway provides NADPH (Ameer et al., 2014). The enzyme that is responsible for catalyzing de novo fatty acid synthesis terminal condensation step is fatty acid synthase (FASN).
Figure 1: *De novo* fatty acid synthesis pathway and sources for FASN substrates.

### 1.2 Fatty acid Synthase (FASN)

There are two types of FASN; FASN I which is a multi-domain enzyme that can catalyze the whole fatty acid synthesis process and is found in the cytoplasm of most eukaryotes (Maier et al., 2008). The other type of FASN is FASN II enzyme system where separate enzymes catalyzed each step of fatty acid synthesis process (White et al., 2005). This group of enzymes is found in the mitochondria of eukaryotes and also in the prokaryotes. The active form of FASN I is composed of a homo-dimer where each monomer has seven different functional catalytic domains (Figure 2). These domains
include the Acyl carrier protein (ACP), which is responsible for substrate channeling from one domain to another; the Ketoacyl synthase domain (KAS) which catalyzes the condensation step; the Ketoacyl reductase (KR) and the Enoyl reductase (ER) which both are responsible for saturating the acyl chain. The Dehydratase (DH) domain is responsible for removing a water molecule from the acyl chain between the two reduction steps. Malonylacetetyl transferase (MAT) which catalyzes the transfer of both malonyl-CoA and acetyl-CoA to the KAS domain, and the Thioesterase domain (TE) which clips the palmitate off the enzyme after reaching the desired acyl-chain length (Maier et al., 2008). FASN I is expressed in a limited number of human adults tissues, including liver, adipose tissue, prostate, cycling endometrium and lactating mammary tissue (Brusselmans and Swinnen, 2009). In certain pathological conditions, FASN is highly expressed, including solid tumors, leukemias and certain types of viral infections (Ameer et al., 2014).
1.3 Regulation of de novo fatty acid synthesis

In general, de novo fatty acid synthesis is regulated by nutritional signals. In the presence of excess amounts of carbohydrate, fatty acid synthesis is highly induced while under low carbohydrate conditions or high polyunsaturated fatty acids conditions fatty acid synthesis is attenuated in liver and adipose tissue (Xu et al., 1999). This control over fatty acid synthesis is achieved at the systematic level by different types of hormones that are released in response to the change in nutrients levels such as insulin, thyroid hormone and glucagon (Blennemann et al., 1992; Haystead and Hardie, 1986; Watkins et al., 1977). Also steroid hormones such as Progesterone and Estrogen can regulate fatty acid synthesis in cycling endometrium and mammary tissue (Chalbos et al., 1990; Escot et al., 1990; Vernon and Flint, 1983). At the molecular level, fatty acid synthesis is
regulated by controlling the activity of Acetyl-CoA Carboxylase (ACC), the rate limiting enzyme in fatty acid synthesis, or modulating the expression levels of the enzymes involved in fatty acid synthesis including FASN. Many metabolites can induce ACC activity such as citrate and glutamate (Boone et al., 2000; Lane et al., 1974), while others inhibit ACC activity such as Coenzyme A and acyl-CoAs (Moule et al., 1992; Ogiwara et al., 1978). The other mechanism by which ACC activity is regulated is through the phosphorylation and dephosphorylation of a number of serine residues (Ser\textsuperscript{79}, Ser\textsuperscript{1200} and Ser\textsuperscript{1215}) which inhibit ACC activity upon phosphorylation mostly by 5’ AMP Activated Protein Kinase (AMPK) (Hardie, 1992). The expression levels of the fatty acid synthesis enzymes are controlled largely by the Sterol Regulatory Element Binding Protein 1c (SREBP-1c) (Ye and DeBose-Boyd, 2011). SREBP-1c is a member of basic helix-loop-helix–leucine zipper (bHLH-Zip) transcription factors family. It is found as an inactive form bound to the endoplasmic reticulum (ER) and is composed of a regulatory domain, transmembrane domain and the bHLH-Zip DNA binding domain (Brown and Goldstein, 1997). SREBPs go through a proteolytic maturation process that is attenuated by sterols, when sterols levels are low the proteolytic maturation leads to the release of the active form to the nucleus (Brown and Goldstein, 1997). Upon translocation to the nucleus, SREBP binds to Sterol Regulatory Element sequence and enhances the expression of fatty acid synthesis enzymes (Horton et al., 2003). Both ACC and SREBP are down stream of many response signaling pathways to several peptide and steroid
hormones including: insulin, thyroid hormone and glucagon (Azzout-Marniche et al., 2000; Lacasa et al., 2001; Mullur et al., 2014; Witters et al., 1988).

1.4 Cancer metabolism

One of the newly accepted hallmarks of cancer is metabolic reprogramming (Hanahan and Weinberg, 2011). Cancer cells need metabolic reprogramming to maintain their high growth rates or to adapt to the change in the microenvironment and the lack of certain nutrients. Elevation in glucose utilization combined with lactate production under aerobic conditions and normal mitochondrial function is one of the early discovered signs of metabolic reprogramming in cancer cells (Warburg et al., 1927). This metabolic reprogramming may have several potential benefits for the cancer cells (Liberti and Locasale, 2016). One of the most likely benefits is the prevention of feedback inhibition generated from the accumulation of NADH and rapid ATP production, leading to uninterrupted flux of glucose into the glycolysis pathway (Pavlova and Thompson, 2016). Another important benefit for lactate production is the effects that lactate has on the tumor microenvironment which includes the induction of angiogenesis (Constant et al., 2000) and the inhibition of T cells and monocytes activity (Fischer et al., 2007; Goetze et al., 2011). Another metabolic alteration that occurs in some types of cancers is the increase in glutamine uptake and catabolism. Glutamine is used to fuel the TCA cycle which in turn provides many intermediates for the synthesis of other amino acids and lipids. In addition, glutamine serves as a substrate for the Solute
carrier family 7 member 5 (SLC7A5) antiport transporter aiding in the uptake of essential amino acids such as leucine which enhances cell protein synthesis through the activation of mTOR pathway (Nicklin et al., 2009). This increase in glutamine utilization is mostly upregulated by c-Myc; which increases the expression of the high affinity glutamine importers SLC1A5 and SN2 (Wise et al., 2008) in addition to enhancing glutaminase expression (Gao et al., 2009). *De novo* fatty acid synthesis is highly induced in many types of cancer (Brusselmans and Swinnen, 2009). This induction of fatty acids synthesis not only provide raw material for the formation of new membranes to support cells proliferation but can also provide the cell with saturated phospholipids that can help in the protection against oxidative stress (Rysman et al., 2010). Cancer cells upregulate lipogenesis through the Sterol Response element binding proteins (SREBPs), which are involved in the increase in the expression of most of the enzymes participating *de novo* fatty acid synthesis (Eberle et al., 2004)(Horton et al., 2002).

### 1.5 FASN as a cancer target

FASN is overexpressed in a variety of cancer types including prostate (Migita et al., 2009; Swinnen et al., 2002; Van de Sande et al., 2005), breast (Menendez et al., 2004a; Milgraum et al., 1997; Wang et al., 2001), colon (Ogino et al., 2007; Zaytseva et al., 2015), lung (Piyathilake et al., 2000) and pancreatic cancer (Bian et al., 2015). The level of FASN expression is correlated with tumor progression, where high FASN expression leads to more tumor aggressiveness and poor therapeutic outcome (Alo et al., 1996; Hamada et
al., 2014; Madigan et al., 2014; Shurbaji et al., 1996; Wang et al., 2004). In contrast to regulation of FASN expression in normal tissues, FASN expression is not inhibited by the increase in fatty acids concentration in circulation or the media of cultured cancer cell lines (Hopperton et al., 2014; Huang et al., 2016). FASN overexpression provides several advantages for cancer cells, they include: (i) providing raw material for the synthesis of new membrane to support the rapid proliferation of cancer cells; (ii) allow the cancer cells more flexibility in the synthesis of the types of phospholipids needed to maintain membranes functions (Rysman et al., 2010), and (iii) increases the ability of cells to produce energy by upregulating cellular respiration pathways (Zaytseva et al., 2015).

Inhibiting FASN activity by pharmacological means or siRNA silencing was found to inhibit cell growth and induce apoptosis in many types of cancer (Pizer et al., 1996a; Ventura et al., 2015; Zhou et al., 2003). Several mechanisms were proposed to explain the ability of FASN inhibition to block cancer cells proliferation and induction of apoptosis. One of the early proposed mechanisms is the deprivation of the cells from fatty acids, which leads to the reduction in phospholipids synthesis and subsequently to blocking cell proliferation (Pizer et al., 1996b). Due to the ability of exogenous fatty acids to rescue a limited number of cancer cell lines from FASN inhibition induced apoptosis, malonyl-CoA was proposed as a mediator of FASN inhibition cytotoxicity (Pizer et al., 2000). Malonyl-CoA is known as an allosteric inhibitor of Carnitine Palmitoyl
Transferase 1 (CPT-1), the rate limiting enzyme in fatty acids oxidation (Witters and Kemp, 1992). Being one of FASN substrates, malonyl-CoA accumulates upon the inhibition of FASN which in its turn leads to the inhibition of CPT-1 which is suggested to increase ceramides levels and induce apoptosis (Bandyopadhyay et al., 2006).

1.6 Current FASN inhibitors

Several groups tried to exploit FASN as a target for cancer by developing inhibitors against the enzyme. One of earliest discovered FASN inhibitors is Cerulenin, which is an antibiotic isolated from the fungus *Cephalosporium caerulens* (Vance et al., 1972). The synthetic FASN inhibitor C75 was developed based on Cerulenin structure and mode of action to overcome Cerulenin instability (Kuhajda et al., 2000). C75, although not selective because of being an activator of CPT-1 (Thupari et al., 2002), was extensively studied and responsible for most of our understanding about the role of FASN in cancer. In a trial to optimize C75 activity, the C93 molecule was developed with lower CPT-1 targeting activity (McFadden et al., 2005). Although C93 showed anti-tumor activity in many tumor Xerograph models (Orita et al., 2007; Ueda et al., 2009; Zhou et al., 2007), the molecule was not advanced into any clinical trials. Many types of polyphenols mainly epigallocatechin gallate (EGCG) and other related compounds have been shown to inhibit FASN activity (Tian, 2006; Wang and Tian, 2001). To overcome the low stability and low potency of these flavonoids, many analogs were generated based on EGCG scaffold (Landis-Piwowar et al., 2007; Puig et al., 2009; Turrado et al., 2012),
that gave rise to a more potent gallate based FASN inhibitor, G28UCM (Oliveras et al., 2010), which although showed better activity than EGCG (Puig et al., 2011), similar to C93, it did not progress into clinical trials.

Another class of FASN inhibitors are the ones targeting FASN thioesterase domain. One of the first molecules discovered in this class is Orlistat which is a pancreatic lipase inhibitor that was approved for weight management but has poor solubility and bioavailability (Kridel et al., 2004). Based on the idea of the drugability of the thioesterase domain, many 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione–based compounds were screened to find inhibitors that has better pharmacological properties than Orlistat (Richardson and Smith, 2007). Although the discovered inhibitors have better toxicity profile than Orlistat, there is no indication that they were verified in vivo (Richardson and Smith, 2007). One of the unique FASN inhibitors is 5-chloro-2-(2, 4-dichlorophenoxo) phenol (Triclosan), this inhibitor is the only one reported to inhibit FASN activity through the binding to an allosteric site on FASN ER domain (Sippel et al., 2014). Some groups tried to employ docking studies to develop FASN inhibitors, the only molecule that resulted from such a strategy is 2-hydrazinoaminothiazole, which is a micromolar inhibitor that binds to FASN KAS domain (Zeng et al., 2011).

One of the most potent FASN inhibitor is BI 99179 (Kley et al., 2011), a compound that has sub-micromolar IC$_{50}$ with blood brain barrier permeability, which may affect feeding behavior and reduce food intake (Loftus et al., 2000). GSK2194069 is a recently
discovered highly potent FASN inhibitor that inhibits β-ketoacyl reductase activity (Hardwicke et al., 2014). Which in contrast to the other potent FASN inhibitor GSK837149A (Vazquez et al., 2008), GSK2194069 is cell permeable but was not tested in vivo yet. AstraZeneca developed a group of FASN inhibitors based on Bisamld scaffold (Bostroem et al., 2008), although some of the Bisamld scaffold derivatives were very potent, they form toxic primary aniline through the cleavage of the secondary amide moiety in rodents plasma (Oslob et al., 2013). One of the potent Bisamld scaffold derivatives AZ22 was found to induce liver steatosis and dermatitis in obese Zucker rats (Wallenius et al., 2008). Based on the same scaffold, potent Imidazopyridine FASN inhibitors were developed (TVB-2640 and TVB-3166) (Oslob et al., 2013). The FASN inhibitor TVB-2640 became the only oral FASN inhibitor advanced to clinical trials for the treatment of advanced solid tumors. Although early phase I open label study results shows that TVB-2640 is tolerated, it induces reversible adverse events in patients such as skin reaction and corneal edema (Patel et al., 2015).
Table 1: Small molecules known to directly inhibit FASN activity

<table>
<thead>
<tr>
<th>Inhibitor name</th>
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<tr>
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<td>EGCG</td>
<td><img src="image2.png" alt="Structure" /></td>
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<tr>
<td>Orlistat</td>
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<td>GSK83714 9A</td>
<td><img src="image4.png" alt="Structure" /></td>
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<tr>
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<td><img src="image5.png" alt="Structure" /></td>
<td>TVB-3166</td>
<td><img src="image6.png" alt="Structure" /></td>
</tr>
<tr>
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<td>BI99179</td>
<td><img src="image8.png" alt="Structure" /></td>
</tr>
<tr>
<td>GSK2194 069</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>AZ22</td>
<td><img src="image10.png" alt="Structure" /></td>
</tr>
<tr>
<td>G 28UCM</td>
<td><img src="image11.png" alt="Structure" /></td>
<td>TVB-2640</td>
<td><img src="image12.png" alt="Structure" /></td>
</tr>
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1.7 Resistance to ErbB2 (HER2) Receptor Tyrosine Kinase inhibitors

HER2 is a member of the ErbB receptor Tyrosine kinase family. The members of this family have three main domains; an extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain (Franklin et al., 2004). Several ligands were found to interact with ErbB2 family members except for HER2 (Falls, 2003). Upon interaction with their ligands, ErbB receptors form dimers that activate their kinase domains except in the case of HER3 which has weak kinase activity (Shi et al., 2010) but can serve as a phosphotyrosine scaffold that leads to the activation of downstream signaling after phosphorylation by other ErbB receptors (Arteaga and Engelman, 2014). The activation of ErbB receptors leads to the stimulation of a network of survival and proliferation pathways steering mainly through PI3K and RAS signaling (Yarden and Pines, 2012). HER2 was found to be amplified in about 30% of breast cancers and is correlated with negative prognosis (Slamon et al., 1987). Based on its importance for cancer progression, many small molecule inhibitors were developed against HER2 for the treatment of HER2+ breast cancer (Schroeder et al., 2014). Lapatinib was one of the early discovered HER2 inhibitors, the molecule is able to block the HER2/EGFR heterodimer phosphorylation and downstream signaling even in the presence of EGF.
(Xia et al., 2002). Although Lapatinib has potent activity, the molecule therapeutic benefits are maintained for a very short time due to the emergence of resistance (Xia et al., 2006). There are many proposed mechanisms that drive resistance against HER2 inhibitors which include: mutations in HER2 tyrosine kinase domain (Li et al., 2014; Wang et al., 2006), recruitment of different signaling pathways that promote survival such as Estrogen receptor signaling (Giuliano et al., 2015; Li et al., 2015; Xia et al., 2006) and heterodimerization with other ErbB family members such as HER3 (Sergina et al., 2007). Based on these mechanisms, one of the most common strategies used to overcome resistance is combination therapy either with new therapeutic agents targeting the HER2 heterodimer (Baselga et al., 2012; Blackwell et al., 2010) or other signaling component of the same pathway (McDermott et al., 2014) and targeting compensatory pathways for acquired resistance (Rimawi et al., 2013). In addition of being one of the key enzymes needed for cancer cells proliferation, FASN overexpression is involved in the mediation of drug resistance in different types of cancer (Bauerschlag et al., 2015; Liu et al., 2008; Liu et al., 2013; Yang et al., 2011). In the case of anti-HER2 drug resistance, FASN inhibition was found to induce apoptosis in resistant breast cancer cell lines (Puig et al., 2011; Vazquez-Martin et al., 2007). The mechanism responsible for the anti-cancer activity of FASN inhibitors is most likely through the attenuation of HER2 expression (Menendez et al., 2004b) by disrupting lipid rafts which can lead to mislocalization of
tyrosine kinase receptors to cell surface or degradation (Menendez et al., 2005; Ventura et al., 2015).

1.8 Rationale and specific aims

That fact that many types of cancer are dependent on de novo fatty acid synthesis and only a limited number of normal tissues have active fatty acid synthesis pathway makes FASN an attractive target for the development of selective cancer therapies. FASN, being a key metabolic enzyme downstream of many growth and survival pathways make it probably less prone to the development of drug resistance through the activation of compensatory pathways. In addition, FASN inhibitors can provide a general means to inhibit tyrosine kinase receptors signaling activity by preventing proper localization of the receptors to the cell surface. This strategy can help in overcoming receptor tyrosine kinase inhibitors resistance.

One of the common themes amongst current FASN inhibitors is a mechanism of action favoring competition with substrate intermediates over cofactor binding. Even in the case of GSK2194069, despite acting on the β-ketoacyl reductase step, the triazolone is only competitive with trans-1-decalone binding and uncompetitive with NADPH (Hardwicke et al., 2014). Inhibitors targeting the FASN co-factor domain therefore remain largely unexplored. Targeting of the substrate domains may in part explain the toxicities and lack of efficacy in vivo of the majority of FASN inhibitors, since in order to act competitively the molecules are lipid like in nature. Three of the FASN enzymatic
activities (ketoacyl reductase, enoyl reductase and malonyl/acetyl transferase) use
purine-containing co-factors in the form of NADPH, acetyl-CoA and malonyl-CoA.
Importantly, inhibitors targeting purine-utilizing enzymes are generally not lipophilic
and have formed the basis of many drugs in clinical use from reverse transcriptase
inhibitors to the newer cutting edge inhibitors targeting protein kinases or heat shock
proteins (Felder et al., 2012; Haystead, 2006; Knapp et al., 2006; Murray and Bussiere,
2009). This indicates that screening for inhibitors targeting the purine interacting
domains of FASN is an appealing strategy for the development of FASN inhibitors.

Although many groups showed that inhibiting FASN activity induces apoptosis,
the mechanism responsible for that is not fully understood especially in the context
where exogenous fatty acids are available to the cells.

In this work, our objective is to fulfil the following aims: (i) Utilize the
fluorescence-linked enzyme chemoproteomic strategy (FLECS) to develop inhibitors
against FASN co-factor binding sites. (ii) Use the resulted inhibitors to probe the
mechanism by which FASN inhibition induces apoptosis in breast cancer cell lines. (iii)
Evaluate the efficacy of such molecule in breast cancer in vivo models.
2. Experimental Procedures

2.1 Identification of the FASN inhibitor HS-106

2.1.1 FELCS screening for FASN inhibitors

Porcine mammary glands were collected from lactating pigs as previously described (Hughes et al., 2012). Tissues were homogenized in lysis buffer A (100mM sodium fluoride, 5mM EDTA, 1mM DTT and 5% glycerol made in 10mM sodium phosphate buffer pH 7.5) in a ratio of 3.0 ml of buffer per each gram of tissue. After removing cell debris by centrifugation at 142,000 x g for 45 minutes and filtering through glass wool, the homogenate was applied to Cibacron blue Sepharose (Sigma Aldrich) pre-equilibrated with buffer B (100mM sodium fluoride, 5mM EDTA, 1mM DTT and 50mM sodium citrate made in 10mM sodium phosphate buffer pH 7.5) in a ratio of 4.5 g of tissue to each ml of settled resin. To remove dehydrogenases and reduce the amount of ATP binding proteins bound to resin, the resin was washed with 10 bed volumes of buffer B then with one bed volume of 5mM NAD made in buffer B followed by one bed volume of buffer B. After that, the resin was washed with one bed volume of 10mM ATP. To label the FASN attached to the resin, to each ml of resin, 1.0 ml of labeling buffer (10mM sodium phosphate buffer pH 7.5 containing 50µg of fluorescein-5-maleimide (pre-dissolved in DMF)) was added to the resin and incubated overnight at 4°C with slow rotation. The resin was then washed with 20 bed volumes of buffer B to remove any excess fluorescein. The resin was suspended in buffer B (1:1, v/v) and
distributed in 96 well filter plates (50μl/well). Fluorescein labeled proteins were eluted from the resin by an in-house library of 3,379 purine-based compounds. For each well, 50μl of each compound was added (1 mM made in buffer B with 10% DMSO). Different concentrations of ATP were used as a control. The eluents were collected in 96 well black plates by centrifugation at 1,260 x g for 5 minutes. Fluorescence in each well was measured at Ex/Em: 485/535nm. Eluents with the highest fluorescent intensity were run on SDS PAGE and proteins were identified by Matrix Assisted Laser Desorption/Ionization (MALDI) Time of Flight/Time of Flight (TOF/TOF) Mass spectrometry (MS).

2.1.2 Proteins identification by MALDI-TOF/TOF MS

Visible bands were excised from the SDS PAGE gels and cut into small pieces approximately 1mm x 1mm. These gel pieces were destained using 1:1 30mM potassium ferricyanide: 100mM sodium thiosulfate for 10 minutes. The destained gel pieces were then washed with 25mM ammonium bicarbonate and acetonitrile alternatively for 5 minutes each wash. This cycle of 5 minute 25mM ammonium bicarbonate wash followed by 5 minute acetonitrile wash was repeated 3 times. The gel pieces were then dehydrated in 100% acetonitrile. After removing all acetonitrile, 25μL of porcine trypsin (Promega) at a concentration of 20 μg/mL was added to the gel pieces. The gel pieces were then kept on ice for approximately 1 hour to allow for the trypsin to remain inactive as it enters the gel. Following this, the gel pieces were incubated at 37°C
overnight (approximately 12-16 hours). Following digestion, the supernatant was transferred to a second tube, and acetonitrile was added to the gel pieces to complete the extraction of digested peptides. This extract was added to the first supernatant and this combined solution, containing the extracted peptides was frozen and lyophilized. The peptides were resuspended in 5 µL of 1:1 acetonitrile: 1% trifluoroacetic acid immediately prior to spotting on the MALDI target plate.

For MALDI analysis, the matrix solution consisted of alpha-cyano-4-hydroxycinnamic acid (Sigma Aldrich) saturating a solution of 1:1:1 acetonitrile: 25mM ammonium citrate: 1% trifluoroacetic acid. Approximately 0.15 µL of peptide solution was spotted on the MALDI target immediately followed by 0.15 µL of the matrix solution. This combined solution was allowed to dry at room temperature. MALDI MS and MS/MS data was then acquired using the ABSCIEX TOF/TOF® 5800 Mass Spectrometer. Resultant peptide mass fingerprint and peptide sequence data was submitted to the UniProt database using the Mascot search engine to which relevance is calculated and protein was identified.

2.1.3 ATP Sepharose binding assay

BT474 cells were seeded at a density of 200,000 cells/ml in Six 15cm plates with DMEM media containing 10% FBS. After 24 hours, the cells were washed with ice cold PBS and incubated on ice for 20min with lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1% Triton and Roche Complete protease
inhibitor). Then the lysate was clarified with centrifugation at 13000rpm for 15 min. The resulting volume (3ml) was diluted with binding buffer (25mM Tris-HCl pH 7.5, 100mM NaCl, 60mM MgCl2, 1mM DTT) (final volume 10ml) and loaded on a 2ml of 50% slurry ATP Sepharose prewashed with the same buffer. Following the incubation at 4°C for 2 hours with rotation, the column was washed with 10ml high salt buffer (25mM Tris-HCl pH 7.5, 500mM NaCl, 60mM MgCl2 and 1mM DTT) followed by 20ml of binding buffer. The resin was re-suspended to 50% slurry with the binding buffer and distributed in 96 well filter plate (50µl/well). To each well, 50µl of ATP, Staurosporine or different HS-106 concentrations was added. After 5 min incubation at room temperature, eluents were collected by centrifugation at 2000rpm for 2min and proteins were separated by SDS-PAGE.

2.1.4 Cell lines

All cell lines used in this work were obtained from ATCC except for HepG2 cell which were a gifts from Dr. Donald McDonnell and the Lapatinib resistant cell lines BT474 and SKBR which were a gift from Dr. Neil Spector and were developed as previously described (Xia et al., 2006).

2.1.5 ³H-glucose and ³H-acetate incorporation into lipids

Incorporation of radiolabeled glucose or acetate in total lipids was measured according to published methods (Chirala et al., 2003). HepG2 cells (80% confluent in 12 wells plates) were starved overnight in MEM Alpha, the medium was then changed
with DMEM medium containing 0.1 g/L glucose, 10% FBS, 5µM insulin and 1 µCi 3-H D-glucose or 1µCi 3-H acetate in addition to different concentrations of each compound. After incubation for 1 hour at 37°C, 5% CO2, the cells were washed with ice cold PBS and detached by treating with 100µl trypsin-EDTA for 10 minutes, followed by adding 1 ml of ice cold PBS. From each well, 1 ml of cell lysate was added to 4 ml scintillation vial and 2 ml of toluene containing 25g/L Butyl-PBD was added. The vials were mixed thoroughly then centrifuged for 30 minutes at 1,600 x g and the 3-H radioactive counts measured by liquid scintillation counting.

2.1.6 Purification of Human FASN

Confluent BT474 cells grown in high glucose DMEM with 10% FBS, were scraped and washed twice with ice cold PBS. Then, the cells (2.26g) were homogenized using potter homogenizer for 5 minutes in 40 ml of buffer A. The homogenate was centrifuged at 142,000 x g for 45 minutes then filtered through glass wool resulting in a volume of 27ml. To the 27ml of homogenate, 6.37 ml of saturated ammonium sulfate (final saturation 20%) was added and mixed slowly for 20 minutes then incubated for 1 hour on ice. After centrifugation at 26,000 x g for 20 minutes, the resulted pellet was discarded and to the supernatant (26 ml), 7ml saturated ammonium sulfate was added (final saturation 35%). After repeating the same procedure in the previous step, the supernatant was discarded and the pellet was collected and dissolved in 20 ml of buffer A and added to 150KDa molecular mass cut off concentrator (Thermo Fisher Scientific,
Waltham, Massachusetts) then centrifuged for 30 minutes at 1,600 x g. The resulting volume on the filter (1.5 ml) was added to a Sephacryl S-300 HR column (1 x 100cm) pre-equilibrated with PBS. The column was eluted with PBS containing 1mM DTT at a flow rate of 0.2ml/min. Fractions (2 ml) were collected and peak fractions were run on SDS-PAGE. Fractions with FASN were identified by MALDI-TOF/TOF MS then pooled and concentrated using 150 KDa cutoff concentrator.

2.1.7 FASN activity assay

Human FASN activity was measured by monitoring the incorporation of 2-[\(^{14}\)C]malonyl-CoA into fatty acids using liquid scintillation counting by a method similar to the one described by Richardson and Smith (2007). FASN (10µg/ml of PBS containing 1mM DTT and 1mM EDTA) was pre-incubated with different concentrations of HS-106 (final DMSO concentration 1%) at 37°C for 30 minutes, then substrates were added (20 µM acetyl-CoA and 200µM NADPH) in a total reaction volume of 100µl. The reaction was initiated by adding 10µl of 50µM malonyl-CoA spiked with 0.05 µCi of 2-[\(^{14}\)C]malonyl-CoA. After incubation for 30 minutes at 37°C, lipids were extracted 3 times with 150µl (2:1, v/v) chloroform: methanol. Then, to the pooled organic phases, 1ml of toluene containing 25g/L Butyl-PBD was added and radioactivity was measured by liquid scintillation counting.
2.2 Probing HS-106 anticancer activity in vitro

2.2.1 Proliferation assay

MCF10A (5,000 cells/well), MCF7(7,500 cells/well), MDA-MB-468(5,000 cells/well), BT474(7,500 cells/well), SKBR3(5,000 cells/well) were seeded in 96 well plates with 10% FBS 4g/L glucose DMEM media except for MCF10A which was DMEM/F12 media. After 24 hours, cells were treated with different concentration of HS-106 or C75. Every 24 hours for five days, media from one of the plates was removed and plate was frozen at -80°C. After collecting all the time points, to each well 100µl ddH₂O was added and the plates were frozen again. Then 100µl of Hoechst 33258 solution buffer (1µl from Hoechst stock (1mg/ml in 1:4 DMSO: H₂O) in 1ml of TNE buffer (10mM Tris, 2 M NaCl and 1mM Na₂EDTA)) was added to each well and fluorescence was measured at Ex/Em: 355/460.

2.2.2 Cell cycle analysis

BT474 cells were treated with different concentrations of HS-106 for 24 hours, cells were collected and fixed with 70% ethanol, washed with PBS then treated with 20mg/ml Rnase A. after that, cells were stained with 50 µg/ml Propidium iodide and DNA content for each cell was quantified using a BD Accuri C6 flow cytometer (BD), and data were analyzed using the CFlow Plus software (BD).
2.2.3 Viability assay

The viability was assayed as described by Niles et al. (2007) using Cathepsin C activity as an indicator for viability. Following BT474 cells treatment with siRNA and HS-106, 50µl of assay buffer (100mM HEPES pH 7.5, 200µM) containing the Cathepsin C fluorogenic substrate Glycine-Phenylalanine-7-Amino-4-Methylcoumarin (GF-AMC) was added to each well. After 45 min of incubation at 37°C, the fluorescence was measured at Ex/Em: 355/460.

2.2.4 Cytotoxicity assay

Cytotoxicity was assayed by measuring released Tripeptidyl peptidase II activity in cells media (Niles et al., 2007). To 50µl of the treated cells media, 50µl of assay buffer (100mM HEPES pH 7.5, 200µM) containing the Tripeptidyl peptidase II fluorogenic substrate Alanine-Alanine-Phenylalanine-7-Amino-4-Methylcoumarin (AAF-AMC) was added. After 8 hours of incubation at 37°C, the fluorescence was measured at Ex/Em: 355/460.

2.2.5 Caspase 3/7 activity assay

The assay was performed using a similar protocol to the one described by Fritz et al. (1998). Cells were seeded at a density of 10000 cells/well and treated with different concentrations of HS-106 or C75. After 24 hours, to each well, 50µl of Caspase assay/lysis buffer (50mM HEPES pH 7.5, 100mM KCl, 5mM EDTA, 10mM MgCl2, 10mM CHAPS, 20% Sucrose, 10mM DTT, 10µM of (Z-DEVD)2-Rh110 (Santa Cruz Biotech) and complete
protease inhibitor (Roche) was added. After 6 hours of incubation at 37°C, fluorescence was measured at Ex/Em: 485/535nm.

2.2.6 Annexin V apoptosis assay

After treating BT474 cells with different concentrations of HS-106 for 24 hours, the Annexin V assay was executed as previously described (Safi et al., 2014). Briefly, cells were collected and stained with Alexa Fluor 488 Annexin V and Sytox Red according to the manufacturer's protocol. Annexin V–positive cells were considered apoptotic, and their percentage of the total number of cells was calculated. Ten thousand events were collected for each sample using a BD Accuri C6 flow cytometer (BD), and data were analyzed using the CFlow Plus program software (BD) and FCS express (De Novo Software).

2.2.7 FASN siRNA transfection

A pool of four species of siRNA against the human FASN gene (ON-TARGET plus Human FASN siRNA SMARTpool) were obtained from Dharmacon. Also ON-TARGET plus Non-targeting siRNA #3 was obtained as a control. DharmaFECT 1 transfection reagent was used for siRNA delivery. siRNAs were diluted in siRNA dilution buffer (Thermo scientific) at a concentration of 5µM. BT474 cells were seeded in 96 well plates at a density of 4000 cells /well in DMEM 10% FBS media. Cells were transfected with target and control siRNA at a concentration of 50nM and 0.1µL/well DharmaFECT 1 transfection reagent following the manufacture protocol. After 48 hours,
cells were treated with different concentrations of HS-106. 72 hours later, the cells were assayed for DNA content, viability and caspase 3/7 activity.

### 2.2.8 Western blot analysis

Cell lysate from cell treated for 24 hour with 10µM of HS-106 or DMSO were loaded (28 µg/ well) and run on Criterion XT Tris-HCl Gel (4–15% gradient) (Bio-Rad) according to manufacture instructions, then the proteins were transferred to PVDF membrane overnight using 25 volt at 4°C. After that, membranes were blocked and blotted for FASN (Cell signaling antibody number 3180) and GAPDH (Cell signaling antibody number 5174).

### 2.2.9 Immunofluorescence staining for surface HER2

Lapatinib resistant BT474 cells were grown on coverslips overnight then treated with different combinations of HS-106 and Lapatinib. After 24 hours, the cells were washed then fixed with 4% PFA/PBS. Successively, cells were incubated in blocking solution (5% goat serum, PBS) for 1 hour. After blocking, cells were incubated with a monoclonal antibody for HER2 at 1:200 (2165, Cell signaling) overnight at 4 °C. The cells were sequentially washed three times with PBS, and then incubated with a goat-anti-rabbit Alexa Fluor 488-conjugated antibody at 1:1,000 (A-11008, Life Technologies) for 2 hours at room temperature. After washing with PBS, Cells were stained with DAPI and imaged using EVOS FL Cell Imaging System.
2.3 Characterization of HS-106 effect on lipids homeostasis

2.3.1 Lipidomics Sample Preparation

BT474 cell were seeded in 15cm dishes at density of 200,000 cells/ml in DMEM 10%FBS. After overnight incubation, the cells were treated with 10µM HS-106 for 2 hours then washed with ice cold PBS and flash frozen. Cells pellets (5 vehicle and 5 treated with 10µM HS-106) were separately thawed on ice, and 100 µL of ammonium bicarbonate, pH 8, was added to each. Pellets were then probe sonicated at power level 3 for 3 bursts of 5 seconds each burst, cooling on ice between bursts. Bradford assay was performed on each solubilized pellet using 10x diluted material. 1 mg from each was taken out and normalized to 137 µL total with AmBic in a 96-well plate. To each sample well, 200 µL of methanol was added followed by the addition of 600 µL of MTBE. The plate was capped and mixed at 800 rpm at room temperature for 1 hour. Plate was then centrifuged at 2000 rpm at room temperature for 10 min and 400 µL of the MTBE/MeOH layer was pipetted out and transferred to another plate. Then the extract was dried under nitrogen gas and samples were reconstituted in 100 µL of 2:1:1 IPA: ACN: H2O. A pool was made by taking an equal volume from all 10 samples.

2.3.2 Mass Spectrometry Lipid Profiling

Each sample was analyzed twice using Ultra performance Liquid Chromatography /Electrospray Ionization/Tandem Mass Spectrometry (UPLC/ESI/MS/MS) in positive ion mode (3 µL) and negative ion mode (10 µL). UPLC
separation was performed using a binary gradient separation on a Acquity UPLC (Waters Corporation, Milford, MA) using a Acquity 2.1 mm x 10 mm 1.7 µm CSH C18 column. Mobile phase A contained 60/40/0.1 v/v/v MeCN/water/formic acid with 10 mM ammonium formate, and mobile phase B contained 90/10/0.1 v/v/v isopropanol/MeCN/formic acid. Lipid separation was performed at 0.6mL/min and 60°C column temperature, using a complex gradient program as follows: initial conditions 40% B, ramp to 43% B at 1.3 minutes, ramp to 50% B at 1.4 minutes, ramp to 54% B at 8 minutes, ramp to 70% B at 8.2 minutes, ramp to 99% B at 12.2 minutes, ramp to initial condition 40% B at 12.3 minutes, then hold at 40% B for re-equilibration until 14 minutes. Via electrospray ionization, the LC eluent was introduced into a G2 Synapt (Waters), and data was collected between 50-1200 m/z in 0.3 seconds; MS/MS was collected at a scan rate of 0.2 sec for peaks above a threshold of 3000 intensity/sec for positive ion and 1000 intensity/sec for negative ion. Source parameters are as follows for positive/negative ion respectively: capillary at 2.7 kV / 2.3 kV, cone voltage of 30 V, 500°C desolvation temperature, 700 L/hr desolvation gas, 150 L/hr cone gas, and a source temperature of 100°C. Lockmass calibration was performed every thirty seconds using a solution of 500 fmol/µL Leucine-Enkephalin in positive (556.2771 m/z) or negative mode (554.2615).

Quantitative data were analyzed in Progenesis QI (Nonlinear Dynamics, Ltd/Waters Corporation). Quantitative data including accurate mass, charge state, retention time
and intensity were exported for additional statistical analysis and have been made available at the link:

(https://discovery.genome.duke.edu/express/resources/3745/3745_IDandStats_HvsD_Pro
genesisQI_062514.xlsx). Putative identifications were made by searching against compiled LipidMaps databases with theoretical fragmentation where available, using 10 ppm precursor and fragment ion tolerance. Putative identifications were confirmed based on accurate mass and retention time using standards for fatty acids myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid using endogenous standards purchased from TCI America, Sigma Aldrich, and Ultra Scientific. A supplemental table containing lipids standards used and observed measurements is shown in appendix F. The Skyline software package

(https://skyline.gs.washington.edu/) was additionally utilized to verify accurate mass, isotope distribution, and quantitative measurements performed by Progenesis QI.

Skyline documents for fatty acid verification may be downloaded at the following link: https://discovery.genome.duke.edu/express/resources/3745/Skyline_FattyAcid_Verification.sky.zip. Skyline document for diacylglycerol verification may be downloaded at the following link:


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2.3.3 Determination of acetate and palmitate incorporation into the main lipids classes

BT474 cells were seeded in 6 well plates in 10% FBS 4.5 g/L glucose DMEM at a density of 400,000 cells/well. After 24 hours the media was changed with 0.1 g/L glucose DMEM containing different concentrations of HS-106. After 1 hour, to each well, 10μCi of 3H acetate or 0.5μCi of 14C palmitate (in complex with BSA) was added and incubated for 1 hour. Then, cells were treated with 500μl trypsin/well for 5 min and subsequently 500μl of ice cold PBS was added to each well. Lipids were separated as previously described (Kaluzny et al., 1985). Briefly, lipids were extracted three times with 700 μl of Chloroform and injected into Sep-PaK Aminopropyl cartridges contains 360mg of resin (Waters) Pre-equilibrated with 10ml chloroform. The cartridges were then injected with 5ml 2:1 chloroform: isopropanol, 2% acetic acid in Ether and methanol to elute neutral lipids, free fatty acids and phospholipids respectively. To each fraction, 1ml of 25g/L Butyl PBD dissolved in Toluene was added and radio activity was measured by scintillation counting.

2.3.4 Lipid droplet staining

BT474 cells were treated with different concentrations of HS-106 under two serum conditions (1% and 10%). After 72 hours, the cells were washed with PBS and fixed for one hour in 10% formalin then incubated in 60% isopropanol for 5 minutes after being washed with water. Subsequently, the cells were incubated for 5 minutes in
oil red O solution (0.3% oil red o in isopropanol diluted 3:2 in water) then washed and stained with Hematoxylin.

2.4 HS-106 in vivo activity

2.4.1 Determination of HS-106 maximal tolerated dose (MTD)

Female FVB/J mice aged to 10-12 weeks (Jackson Labs, Maine) were intraperitoneally (IP) injected twice weekly with HS-106 at the described doses. Mice were monitored for signs of toxicity by standard Mouse Phase 1 Unit (MPIU; https://www.med.unc.edu/mousephase1) protocols and approved by UNC-CH IACUC. Prior to end of the study 150 µl of whole blood was drawn via submandibular bleed and used to determine hematology values, liver, and kidney functions by HemaTrue Hematology Analyzer (HESKA, Loveland, CO,USA) and VITROS® 350 Chemistry System (J&J, New Brunswick, NJ) according to manufacturer protocols. To determine the long term effects of HS-106 on mice weight, female FVB/J mice aged 12-16 weeks were treated with the indicated concentrations of HS-106 twice weekly by IP injection for 60 days. Mice body mass was assessed weekly, and mice were observed daily for signs of toxicity (e.g. labored breathing and hunched posture).

2.4.2 Determination of HS-106 efficacy in vivo

Single-time parous female MMTV-NEU mice (Jackson Labs Strain 002376) were used to test the efficacy of HS106 (15 mg/Kg, IP, BIW) alone and in combination with Carboplatin (50 mg/Kg, IP, QW). Mice were monitored for tumor development by
palpating them weekly as per UNC Lineberger Mouse Phase 1 Unit protocol. Once tumors were observed, the mice were placed on treatment. The tumor-bearing mice were injected weekly with HS-106 and/or Carboplatin. The solvent for HS-106 consists of 50% dimethyl sulfoxide (DMSO) and 50% saline (0.9% sodium chloride solution). Clinical grade Carboplatin was purchased from the UNC Hospital pharmacy. Tumor volume was measured at the time of injection by caliper and width (short diameter) and length (long diameter) in millimeters (mm) were recorded. The volume was calculated using the formula: length x width2 x 0.5. At the time of injection, body composition was assessed and weight measurements (in grams) were recorded and used to determine toxicity. After three weeks, tumor progression was calculated using the formula: (21 day volume – initial volume)/initial volume x 100. This percent change in tumor volume, at 21 days, was used to assess the objective response rate of the therapies. Mice were treated and monitored until euthanized due predetermined humane endpoints per UNC IACUC protocol 13-190. Overall survival was calculated by date of necropsy – initial treatment date. The same protocol was used for the assessment of HS-106 and HS-106 Carboplatin combo in the Triple-negative breast cancer GEMM, C3Tag mouse model.

2.4.3 HS-106 pharmacokinetics

MMTV-NEU mice were IP injected with 15mg/kg of HS-106, after different time points (0, 5min, 1hr, 4hr, 8hr and 24hr), tissues and blood were collected from each animal. Whole blood was centrifuged and only the plasma was retained. Whole liver,
kidneys, brain, spleen and tumor were frozen and stored at -80°C until processing. Before quantifying HS-106 in the plasma and tissue, a standard curve was made using HS-106 and HS-427 (a close structural analogue to HS-106) as an internal standard. HS-106 and HS-427 were diluted in FBS and mixed with four volumes of acetonitrile. The resulting solution was then filtered through a 0.2μm Polytetrafluoroethylene (PTFE) membrane (VWR, Radnor, PA). LCMS analyses were performed on an Agilent 2100 LC system (Santa Clara, CA) connected to an Agilent 6311 Ion Trap Liquid chromatography–mass spectrometry (LCMS). Samples were injected (15 μL injection volume) onto an Agilent Eclipse Plus C18 column (4.6 mm i.d. × 150 mm, 5 μm particle size) at room temperature. Mobile phases A (0.2% formic acid in water) and B (0.2% formic acid in acetonitrile) were mixed to form a gradient of 5%-100% B over 9 minutes. MS analysis was performed in positive ionization mode (Nebulizer 50 psi, Dry Gas 12.0 L/min, Dry Temperature 350 °C, Capillary 31 nA, Ramp Range 4.5-1.5 kV). The detection mass target was 335 m/z and the scan range was 100-1000 m/z. Data was analyzed on Bruker Data Analysis software for 6300 Series Ion Trap v.4.0 sp2. Extracted ion chromatograms (EIC) were generated for target masses [M+H]+, m/z 339 and [M+H]+, m/z 335 and peak areas were determined using standard integration protocol or manual integration. The total ion chromatogram (TIC), UV chromatogram, and mass spectra (MS) were also obtained for each run. The area under the curve from the EIC for HS-106 [M+H]+, m/z 339 and for HS-427 [M+H]+, m/z 353 was determined. The EIC area ratio
for HS-106 compared to HS-427 was calculated and this ratio was used to plot a standard curve based on the known concentrations of HS-106.

The plasma samples were processed by diluting 1:4 in acetonitrile, and filtered through a 0.2μm PTFE membrane. 15μL of the solution was run through an Eclipse Plus C18 column and analyzed by an Agilent Ion Trap 6130 LC-MS. The resulting EIC ratios from HS-106 compared to HS-427 were then determined, which was used to calculate the concentration of HS-106 in the diluted sample.

The final concentration of HS-106 in plasma was calculated per mL of plasma. The tissue samples were weighed and homogenized in ddH₂O. The resulting homogenate was then processed and analyzed in the same manner as the plasma samples. The resulting EIC ratios from HS-106 compared to HS-427 were then determined, which was used to calculate the concentration of HS-106 in the diluted sample. Final concentration of HS-106 in the tissues was calculated per gram of tissue using the weight of each tissue measured before sample processing.
3. Modified FLECS screen resulted in the discovery of a thiophenopyrimidinone based FASN inhibitor HS-106

3.1 Purine nucleotides elutes FASN from Cibacron blue Sepharose

To specifically identify inhibitors of FASN targeting its nucleotide binding pockets, we utilized Cibacron 3GA Sepharose. This medium has been previously used to purify NAD and NADP binding proteins from crude tissues extracts (Miyaguchi et al., 2011; Muratsubaki et al., 1994). FASN enriched extract from lactating pig mammary gland was bound to the resin and labeled with the cysteine reactive fluorescein-5-maleimide. The purine nucleotides ATP and NADPH were able to elute FASN and other proteins in a dose dependent manner with more FASN eluted by NADPH than ATP, which elutes more proteins as reflected by the fluorescence measurements (Figure 3A).

![Figure 3: NADPH and ATP elution of fluorescently labeled pig mammary gland proteins from Cibacron blue Sepharose. (A) Fluorescence measurement of proteins eluted by different concentrations of NADPH and ATP from Cibacron blue sepharose. (B) The eluted proteins were separated by SDS-PAGE and identified by MALDI-TOF/TOF MS (* identified FASN).](image-url)
3.2 Several FASN elutors resulted from Cibacron blue Sepharose FLECS based screen

Having established that fluorescein labeled FASN could be competitively released from the Cibacron blue Sepharose resin with adenine containing nucleotides (Figure 3), we subsequently screened the bound enzyme against a single concentration of an in-house small molecule library comprising compounds with structural similarity to any purine or known purine analog scaffold (Carlson et al., 2013).

Figure 4: FLECS screen schematic. The screen involves loading Cibacron blue Sepharose with lactating pig mammary gland homeogenate following washing, and labeling of the bound proteins with fluorescein. Fluorescein labeled proteins are competed off Cibacron blue resin with purine based small molecule library. Proteins from the eluants that has high fluorescence intensity are separated by SDS-PAGE and silver stained then identified by mass spectrometry.
Of the 3,379 compounds screened, 247 were found to yield a fluorescent signal at Ex/Em: 488nm/522nm (Figure 6). One hundred and fifty-five of the molecules selectively eluted FASN from the resin, and 20 candidate lead compounds were progressed according to their FASN selectivity (assessed with SDS-PAGE, silver staining and mass spectrometry). These 20 compounds were reduced to 13 molecules based on the absence of any obvious chemical liabilities (Figure 5). Next, the molecules were tested for their ability to inhibit FASN activity in a HepG2 cells using an assay that measured the incorporation of $^3$H glucose into lipids (Figure 5).
Figure 5: *in vitro* activity of top screen hits. (A) Molecules activity in the $^3$H glucose incorporation into lipids. (B) Structure of each molecule. (C) Elution profile of each molecule from blue Sepharose.
Identified proteins:
1,5,10,14,17,20,22,25,28,31,34,37,41 FASN
2,6,11,15,23,26,29,32,38,45 Argininosuccinate synthetase
1,3 Histidine-rich glycoprotein
4,8,13,16,19,21,24,27,30,33,36,40,43 Eukaryotic translation elongation factor 1 alpha
1,7,12,18,35,39,42 Albumin
9,44 Medium-chain specific acyl-CoA dehydrogenase, mitochondrial isoform 2

Figure 5 (Continue).
Of the 13 molecules tested, the thiophenopyrimidine molecule HS-106 was the most potent inhibitor of the incorporation of tritiated glucose into lipids.

![Diagram of the FLECS screen for FASN inhibitors]

**Figure 6: Summary of the FLECS screen for FASN inhibitors.** The screen of 3,379 purine-based compounds identified 247 hits with high fluorescent signal. Of the 247 hits, 155 were selected by the virtue of both a high FASN-intensity and a low number of non-FASN protein bands. The 20 most selective compounds were tested for anti-FASN activity in a $^3$H glucose incorporation assay and the molecule with the highest activity (HS-106) was selected to be further studied as a lead molecule.
3.3 HS-106 a thiophenopyrimidine that inhibits FASN activity

To confirm that HS-106 elution of FASN from Cibacron blue Sepharose and inhibition of glucose incorporation into lipids is due to HS-106 direct inhibition of FASN activity, we tested the ability of HS-106 to inhibit purified human FASN activity. Radioactive malonyl-CoA incorporation into palmitate was chosen as a direct method to assess FASN activity by liquid scintillation counting. FASN was purified from BT474 cells to a purity of around 85% (Figure 7A) and 10µg of FASN was used in each individual assay. HS-106 ability to inhibit $^{14}$C malonyl-CoA incorporation into lipids was tested at different concentrations of HS-106 and the IC$_{50}$ was found to be around 3.71µM (Figure 7D).
Figure 7: Characterization of purified FASN and HS-106. (A) Purity of FASN from BT474 cells was assessed by SDS-PAGE; lane 2 and 3 is 20μg and 10μg of loaded protein respectively, estimating the purity to be around 85%. (B) Increase of purified FASN concentration shows a dose response increase in the activity of $^{14}$C malonyl-CoA incorporation into lipids. (C) Inhibitory activity of C75 was measured in the same assay. (D) HS-106 inhibits purified human FASN activity of $^{14}$C malonyl-CoA incorporation into lipids with an IC$_{50}$ of 3.71 μM.
Due to the involvement of a large number of enzymes in the incorporation of glucose into lipids, acetate was used as a tracer to test HS-106 ability to inhibit fatty acid synthesis. There are only two enzyme steps between acetate and palmitate synthesis which are the ligation reaction catalyzed by acetyl-CoA synthetase producing acetyl-CoA and the carboxylation reaction catalyzed by acetyl-CoA carboxylase which leads to malonyl-CoA formation. HS-106 was potently able to block both acetate and glucose incorporation into total lipids, with IC\textsubscript{50} values of 147 nM and 213 nM, respectively in HepG2 cells (Figure 8).

![Graph showing inhibition of acetate and glucose incorporation into lipids by HS-106.](image)

**Figure 8: in vitro activity of HS-106.** HS-106 inhibits both tritiated acetate (IC\textsubscript{50} 147 nM) and glucose (IC\textsubscript{50} 213 nM) incorporation into lipids in HepG2 cells.

HS-106, being a racemic mixture, we decided to test the activity of each enantiomer of HS-106. Both HS-106 enantiomers (HS-79 and HS-80) were synthesized in addition to a truncated version of HS-106 (HS-102) as a negative control (Figure 9A). We tested the ability of these molecules to elute fluorescein labeled pig mammary protein off the blue Sepharose resin. There was no significant difference between them in the intensity of the
fluorescence of the eluted proteins or the intensity of the FASN band except for HS-102 which did not show any activity. The molecules were also tested in the acetate incorporation assay in BT474 cells. Both of the HS-106 enantiomers were able to inhibit the incorporation of tritiated acetate (IC₅₀ 5.84 μM, 1.57 μM and 7.13 μM for HS-106, HS-79 and HS-80 respectively) into lipids. The truncated molecule HS-102 did not have any effect on acetate incorporation.
Figure 9: HS-106 enantiomers activity *in vitro*. (A) Structures of HS-106 enantiomers. (B) Cibacron blue Sepharose was incubated with porcine lactating mammary gland extracts and bound proteins were labeled with fluorescein, the labeled proteins were eluted with different concentrations of HS-106 enantiomers, the eluted proteins fluorescence were measured. (C) The eluted proteins were separated by SDS-PAGE and proteins were identified by MS (* FASN). (D) Both HS-106 enantiomers were able to inhibit the incorporation of tritiated acetate (IC50 5.84 μM,
1.57 μM and 7.13 μM for HS-106, HS-79 and HS-80 respectively) into lipids in BT474 cells while the truncated molecule HS-102 showed no effect on acetate incorporation.

To assess the selectivity of HS-106, we surveyed data derived from prior screens against our in house library. None of the previously screened proteins (ACC, Hsp90, Hsp70, TRAP-1, DAP kinase 3(ZIPK), IRAK 2, AMPK alpha and gamma subunits, NEK9, dengue nonstructural protein 5 (NS5) malarial kinase PfPK9, and HSF-1) were targeted by HS-106.
Figure 10: Selectivity of HS-106. Individual compounds were assayed for their ability to elute proteins from Cibacron blue resin. Blue-red color spectrum indicates protein concentration, as measure by fluorescence in the FLECS screen. SDS-PAGE and mass spectrometry analysis showed that HS-106 selectively elutes FASN compared with strong (HS-206160) and weak (HS-202889) hits. The selectivity plot (red graph). Compound library was screened for inhibitory activity against the following enzymes: ACC, ZipK, AMPKα, AMPKγ, TRAP1, HSP70, NS5, and IRAK2; HS-106 was only active against FASN.
In addition, we tested the ability of HS-106 to elute proteins of ATP Sepharose loaded with BT474 lysate and found that HS-106 does not elute any proteins over that observed with the vehicle DMSO (Figure 11), further confirming selectivity.

Figure 11: HS-106 elution of ATP Sepharose bound proteins. Increased concentrations of HS-106 (DMSO, 1, 2.5, 5 and 10mM lane 1-5 respectively) does not elute any proteins more than the DMSO from ATP Sepharose loaded with BT474 cells lysate. Lane 6 and 7 are 10mM Staurosporine and 50mM ATP, respectively as positive controls.

3.4 Discussion

Many high throughput assays were developed to screen for FASN inhibitors. Most of these assays suffer from the need for specialized equipment (Vazquez et al., 2006; Weiss and Glickman, 2003), radioactive tracers (Bays et al., 2009; Weiss and Glickman, 2003) and highly pure enzyme. The FELCS screen provides a simple method for the identification molecules interacting with FASN purine binding domains. Three of the FASN enzymatic activities (ketoacyl reductase, enoyl reductase and malonyl/acetyl transferase) use purine-containing co-factors in the form of NADPH, acetyl-CoA and
malonyl-CoA. The binding pockets for these cofactors provide possible interaction sites for molecules screened by the blue Sepharose method. There was no detectable difference in the elution profile between the HS-106 enantiomers. This indicate that, although the blue Sepharose screen is a valuable hit identification method it may not be robust enough for lead optimization, unless if it’s used to filter out inactive and non-selective molecules. Another minor disadvantage of this method is the high initial hit rate which makes the use of a secondary screen or prioritization method is very important. Although, the use of tissue extract instead of the purified enzyme can provide an advantage over the other screening methods, it can be a source for variation between the different batches of tissues.

While we did not see any of the enantiomers being completely inactive, the IC₅₀ of HS-106 in the acetate incorporation assay seems to be in between that of HS-79 and HS-80, which is consistent with the behavior of a racemic mixture. We believe that having all the molecules within the same range of IC₅₀ (between 1 and 10µM) is due to the presence of the 5-membered ring in the middle of the molecules which can provide some type of flexibility for the overall geometry of the molecule making all of the enantiomers to have similar binding behavior.

HS-106 is a unique thiophenopyrimididine that was not reported before to have any FASN activity or any other enzyme inhibitory activity. The molecule can be readily synthesized in one efficient step from the chloropyrimidine and amine starting
materials (Appendix A). The synthetic route to HS-106 can readily be adapted for the preparation of analogs that are similar in structure, suggesting that the pharmacological properties of HS-106 can be improved.
4. HS-106 elicits anti-proliferative activity in breast cancer cell lines by the induction of apoptosis and altering cellular lipids homeostasis

4.1 HS-106 inhibits proliferation in several breast cancer cell lines

To evaluate the potential of HS-106 in breast cancer, we first tested its effects on proliferation across a panel of non-tumorigenic (MCF10A) and aggressive breast cancer cell lines including ER+ (MCF7), triple negative (MDA-MB-468) and HER2+ (BT474 and SKBR3). HS-106 inhibited the proliferation of aggressive cell lines with similar potency to C75, but showed lower activity in the non-tumorigenic cell line MCF10A (Figure 12A-E). The weaker effects of HS-106 in MCF10A cells correlated with low expression of FASN in this cell line relative to the more aggressive lines, suggesting that MCF10A are less dependent on FASN for growth (Figure 12F). HS-106 treatment of BT474 cells did not induce cell cycle arrest except for an increase in the Sub 2N cell population (Figure 12G).
Figure 12: Anti-proliferative activity of HS-106. (A-E) Based on the DNA content measured by staining with Hoechst, treating various types of breast cancer cell lines with one dose of 50µM of HS-106 (green circle) was able to inhibit cells proliferation with similar potency of 50µM C75 (red triangle) except for the non-tumorigenic cell line MCF10A when compared to control (blue squares). (F) Treating breast cancer cell lines with 50µM of HS-106 for 24 hours did not have any effect on the expression of FASN. (G) Cell cycle analysis for BT474 cells treated with different concentrations of HS-106 for 24 hours shows an increase in the Sub 2N population.
4.2 Anti-proliferative activity of HS-106 is due to the induction of apoptosis

Inhibition of FASN in rapidly proliferating breast cancer cells would be predicted to have two major effects; first, limit the oxidative capacity of the mitochondria through increasing malonyl-CoA levels; second, trigger programmed cell death pathways via accumulation of ceramides. To investigate the latter mechanism, we examined Caspase 3/7 activation in response to HS-106 and C75 treatment (Figure 13).

Figure 13: HS-106 induce apoptosis in HER2+ breast cancer cell lines. (A) The indicated cells were treated with different concentrations of HS-106 or C75 for 24 hours then the Caspase 3/7 activity was assayed using the fluorogenic substrate (DEVD)\_2-r110. (B) The same experiment was repeated for longer treatment time (48 hour) further showing that HS-106 has more selective induction of apoptosis in the HER2+ cell lines when compared with C75.
Consistent with their tumorigenic capacities, SKBR3 and BT474 cells had 2 to 10 fold (respectively) higher caspase activity than MCF10A cells in response to HS-106 or C75 treatment. The ability of HS-106 to induce apoptosis was also confirmed by detecting the presence of phosphatidylserine and phosphatidylcholine on the outer leaflet of the plasma membrane using fluorescently labeled Annexin V and flow cytometry (Figure 14).

![Graph showing apoptosis induction](image)

**Figure 14:** HS-106 induces apoptosis in BT474 cells. BT474 cells were treated with different concentrations of HS-106 for 24 hours, fixed, labeled with fluorescent Annexin V antibody and stained with Sytox red viability dye. HS-106 was found to increase the apoptotic cells population and the ones going through secondary necrosis.

To determine if the toxic effect of HS-106 in the breast cancer cells is due to the direct inhibition of fatty acid synthesis, we tried two different approaches; silencing the expression of FASN and rescue the cells by manipulating the concentrations of FASN substrate and product. First, to test the effect of silencing the expression of FASN on the ability of HS-106 to induce apoptosis, we tried to knockdown FASN expression by a
pool of highly specific siRNA against different sequences in the FASN gene (Figure 15A). After transfecting BT474 cells with FASN siRNA pool, which was able to reduce the expression by 85%, the cells were treated with different concentrations of HS-106. The potency of HS-106 in the reduction of cell viability and the induction of apoptosis were found to decrease after the siRNA treatment. This may indicates that the cytotoxic effect of HS-106 is due to the direct inhibition of fatty acid synthesis.
Figure 15: Effect of FASN knockdown on HS-106 induced apoptosis. (A) BT474 cells were treated with different concentrations of FASN smartpool siRNA, 85% of reduction in FASN expression was observed. (B) FASN siRNA were found to induce apoptosis and inhibit cell proliferation in BT474 cells. BT474 cells were treated with different HS-106 concentrations after FASN siRNA transfection; FASN siRNA transfection was able to reduce HS-106 ability to (C) induce Caspase 3/7 activity, (D) inhibit cells proliferation and (E) viability.

To further confirm that HS-106 induction of apoptosis is directly related to the inhibition of FASN, we tried to rescue the breast cancer cells by pretreating them with
different combinations of fatty acids (palmitate and oleate) and the ACC inhibitor 5-(Tetradecyloxy)-2-Furoic Acid (TOFA) to prevent malonyl-CoA accumulation (Figure 16). However, in our hands, only TOFA treatment was able to completely reverse the effect of HS-106 in BT474 cells which was not due to a general anti-apoptotic activity of TOFA (Figure 17), while oleate and palmitate mixture, or the combination of both the fatty acids mixture and TOFA, did not fully reverse the effect of the inhibitor. In SKBR3 cells, TOFA, fatty acids mixture and the combination of both, was able to partially reverse the effect of HS-106.

Figure 16: Reversal of HS-106 induced apoptosis by fatty acids and ACC inhibition. Cells were pretreated for one hour with fatty acids mixture (FA) made as a mixture of 1:2 palmitate/oleate in complex with 0.1%BSA or 15μM TOFA or both (TOFA+PA). All the treatments contained the exact amount of BSA and DMSO. Then, cells were treated with different concentrations of HS-106 or C75 for 24 hours and Caspase 3/7 activity was measured.
This indicates that HS-106 induces apoptosis mostly by increasing the concentration of malonyl-CoA rather than depriving cells from palmitate.

Figure 17: TOFA effect on Staurosporine induction of apoptosis. Pretreating BT474 cells with TOFA does not block Staurosporine induced apoptosis.

4.3 HS-106 has profound effects on the lipidome

To determine the effects of HS-106 on the whole cell lipid profile, we carried out lipidomic analysis by LC/MS-MS following 2 hours of exposure to 10µM HS-106 in BT474 cells grown in full serum media. Using ESI+ and ESI- profiling, more than 3000 lipids features can be simultaneously quantified which showed significant changes in the abundance of many lipid species between vehicle and treatment. Our analysis showed that HS-106 induced more than two fold change in the abundance of 167 specific molecules (p < 0.01 relative to vehicle). Of the lipids that were identified several ceramides, diacylglycerols and fatty acids were found to increase upon treatment with HS-106 (Figure 18B).
Figure 18: HS-106 effects on the Lipidome. BT474 cells were treated with 10 μM of HS-106 for two hours and then lipids were extracted and subjected to LC/MS-MS. More than 3000 lipid features were quantified using both ESI+ and ESI- analyses. (A) Each point represents one of the lipid molecules that were quantified, aggregated for ESI+ and ESI-. The color of each dot represents how significant is the difference in the abundance between the control and treatment. (B) Of the lipids that were identified, diacylglycerols, ceramides and fatty acid were found to increase over the control (P value * < 0.01, # < 0.05, n=5).
4.4 **HS-106 induces neutral lipids droplet formation**

The increase in the abundance of many fatty acids over the control, most significantly the polyunsaturated lipids (Figure 18B), suggests a compensatory effect as a result of their uptake from the full serum media. Diacylglycerols were also found to increase significantly, which can indicate an overall increase in the lipolysis of Phosphatidylinositol 4,5-bisphosphate (PIP2) or an increase in *de novo* synthesis of diacylglycerols. Increase in diacylglycerol accumulation would be expected as a consequence of FASN inhibition, since this would be predicted to promote accumulation of glycerol, a precursor of triglyceride and diacylglycerols. This is because the flux of carbons normally supplied by glycolysis for *de novo* fatty acid is now blocked at the level of FASN itself causing accumulation of all upstream intermediates (Haystead et al., 1989). This was confirmed by a $^{14}$C palmitate uptake assay where HS-106 treatment increased $^{14}$C labeling of free fatty acids (Figure 19A). We were able to confirm the accumulation of neutral lipids by performing an Oil red O stain for lipids droplets under different serum conditions showing an increase in lipid droplet formation when BT474 cells are exposed to HS-106 in full serum (Figure 19C).
Figure 19: HS-106 effect on palmitate uptake and neutral lipids formation. (A) BT474 cells were treated with different concentrations of HS-106 for 1 hour then lipids were separated by aminopropyl cartridges after incubation the cells with $^{14}$C palmitate for two hours. The results show a dose dependent increase in palmitate sequestering into free fatty acids and reduction in its incorporation into phospholipids. Similar to free fatty acids, neutral lipids increase except for at 50μM HS-106 where they decrease. (B) A similar experiment was done with $^3$H acetate as a tracer which showed that HS-106 was able to inhibit the incorporation of acetate into the different types of lipids, especially the more abundant phospholipids. (C) Treatment of BT474 cells with different concentrations of HS-106 at 10% FBS conditions, induce the formation of lipids droplets as shown by oil red O staining indicating an increase in neutral lipids formation when compared with 1% FBS.
4.5 ceramides accumulation could be partially responsible for HS-106 induction of apoptosis

Other lipids of particular note that significantly increased with HS-106 treatment are ceramides, which are considered as pro-apoptotic signaling lipids (Haimovitz-Friedman et al., 1997). The increase of ceramides would be expected due to malonyl-CoA (the direct substrate of FASN) accumulation and its effects on CPT-1 inhibition (Bandyopadhyay et al., 2006). As a consequence, any free fatty acids (derived primarily from the extracellular media) are likely to be condensed to 3-keto dihydrosphingosine and on through a series of reduction and acylation steps to various ceramides such as dihydroceramide and ceramide.

We tried to go further into verifying if ceramides accumulation as the main cause of apoptosis by pretreating BT474 cells with different ceramides synthesis inhibitors targeting various enzymes in both the de novo and salvage ceramides synthesis pathway (Figure 20).
Figure 20: HS-106 induces apoptosis in BT474 cells that can be partially reversed by the SPT-1 inhibitor Myriocin. Several ceramide synthesis inhibitors were used to rescue BT474 cells from apoptosis, the schematic shows the positions of the
enzymes targeted by these inhibitors in the de novo and salvage pathways. Before treating BT474 cells with increasing concentrations of HS-106, the cells were treated with 10μM of each inhibitor and caspase 3/7 assay was performed after 24 hours.

None of the ceramides synthesis inhibitors were able to reverse HS-106 induced apoptosis except for Myriocin which is a serine palmitoyltransferase inhibitor was able to partially reverse apoptosis. This Indicates that ceramides accumulation may not be the only rout by which HS-106 induces apoptosis.

4.6 Discussion

HS-106 shows anti-proliferative activity in a variety of breast cancer cell lines. In MCF10A cells which are non-tumorigenic breast epithelial cells, HS-106 has lower activity when compared to the well-known fatty acid synthase inhibitor C75. This testimonial for selective toxicity is mostly due to the lower dependency of MCF10A cells on de novo fatty acid synthesis. As we show that the anti-proliferative activity is due to the induction of apoptosis, we tested if lowering FASN expression in BT474 would reduce the efficacy of HS-106 in a similar way to what we see in the MCF10A cells. Due to the inability to get a 100% transfection efficacy, the siRNA was not able to completely block HS-106 toxicity because of the residual FASN expression.

Blocking malonyl-CoA accumulation by inhibiting ACC activity was able to reverse HS-106 induced apoptosis. This indicates that HS-106 induced apoptosis is due to malonyl-CoA accumulation which is known to inhibit CPT-1 activity (McGarry et al., 1983), which connects FASN inhibition to apoptosis through the increase in ceramides
concentration (Bandyopadhyay et al., 2006). The accumulation of ceramides was confirmed by our lipidomics analysis, which may indicate in addition the inhibition of CPT-1, an induction of sphingomyelinase activity. To find out which pathway is responsible for the increase in ceramides concentration, different inhibitors of ceramides accumulation were used to block HS-106 induced apoptosis. The inability of the ceramides salvage pathway inhibitors to rescue the HS-106 induced apoptosis indicates that ceramides accumulation is unlikely due to an increase in sphingomyelinase activity. In addition, the partial rescue by Myriocin suggests that there is another signal that contributes to apoptosis downstream of the blockage of fatty acids oxidation.

HS-106 treatment was also found to induce an increase in diacylglycerols and unsaturated fatty acids abundance. There are two main pathways by which diacylglycerols can be formed; by de novo synthesis from glycerol and fatty acids, which increases when there are large quantities of these precursors; and from the lipolysis of PIP2. Based on our findings with HS-106, the former pathway would be favored since inhibition of the fatty acid synthesis pathway in general leads to the glucose being diverted into the synthesis of glycerol (Haystead et al., 1989). When combined with the uptake of fatty acids from the media and the inhibition of CPT-1, these conditions will favor an increase in diacylglycerols abundance. The palmitate uptake experiment and the lipids droplets staining at different serum concentrations confirms the previous finding by showing the partitioning of palmitate into neutral lipids instead of
phospholipids. This explains the inability of palmitate to completely rescue HS-106 induced apoptosis. Collectively, these data therefore reveal why FASN inhibition would induce anti-proliferative activity in vivo in spite of the fatty acids provided from the circulation as previously reported in literature. The observation of the partitioning of exogenous fatty acids into neutral lipids instead of phospholipids is noteworthy because it may explain the induction of steatosis in high fat fed Zucker rats treated with the FASN inhibitor AZ22 (Wallenius et al., 2008). With the increase in adapting ketogenic diet by cancer patients as an adjuvant therapy (Allen et al., 2014), it would be essential to conduct more studies about the effect of FASN inhibition and nutrition on the functions of normal lipogenic tissues. Such type of diet would introduce large amounts of fatty acids and protein to the circulation, taking into account that glutamine from the protein can fuel glyceroneogenesis (Nye et al., 2008). This may increase neutral lipids formation in lipogenic tissues while FASN activity is inhibited.

Combining the accumulation of ceramides and neutral lipids in addition to the inhibition of phospholipids formation by blocking of de novo fatty acid synthesis all together, could induce changes in the plasma membrane composition. The uptake of exogenous unsaturated fatty acids while de novo fatty acid synthesis is inhibited would limit the cell ability to produce phospholipids with saturated acyl chains which are important for membranes functions (Rysman et al., 2010). While ceramides are known to displace cholesterol from lipid rafts which may affect lipid rafts structure and function.
(Megha and London, 2004). These potential changes in lipid rafts structures is noteworthy due to its effect on the localization of growth factors receptors to the cell surface especially HER2 in the case of BT474 cells.
5. HS-106 re-sensitizes Lapatinib resistant cell lines by disrupting HER2 and HER3 expression

After we established the anti-cancer activity of HS-106 in breast cancer cell lines, we assessed the potential of HS-106 in the treatment of drug resistant breast cancer cell lines by testing the anti-cancer activity of HS-106 in Lapatinib resistant HER2+ breast cancer cell lines.

5.1 HS-106 Acts Synergistically with Lapatinib

First, we tried to confirm HS-106 ability to inhibit fatty acid synthesis in Lapatinib resistant cell lines. HS-106 was able to inhibit the incorporation of tritiated acetate into lipids in both Lapatinib resistant BT474 and SKBR3 cell lines (Figure 21).

![Figure 21: HS-106 inhibition of acetate incorporation into lipids in HER2+ Lapatinib resistant cell lines.](image)

HS-106 inhibition of fatty acid synthesis in the Lapatinib resistant cell lines was found to attenuate cells proliferation. When combined with Lapatinib, HS-106 was found to have a synergistic effect on cells proliferation (Figure 22).
We tried to assess the effect of HS-106 on Lapatinib induced apoptosis in the resistant cell lines. We treated both Lapatinib resistant BT474 and SKBR3 cells with increased concentrations of different Lapatinib and HS-106 combinations. Then, we performed caspase 3/7 activity and cytotoxicity assays. Treating with HS-106 seems to increase the sensitivity of the resistant cell lines to Lapatinib. Adding as low as 1µM of HS-106 to Lapatinib treated cells increased the caspase 3/7 activity by three fold over the cells treated with Lapatinib alone, while increasing the HS-106 concentration makes the cells to go rapidly through apoptosis to secondary necrosis as shown by the cytotoxicity assay results (Figure 23).
Figure 23: HS-106 re-sensitizes Lapatinib resistant cell lines.

The synergistic effect of HS-106 and Lapatinib was confirmed by performing Annexin V assay in Lapatinib resistant BT474 cells. Similar to the caspase 3/7 assay, treating cells with low concentrations of HS-106 synergize the effect of Lapatinib and increase the population of cells going through apoptosis and secondary necrosis (Figure 24).
Figure 24: Lapatinib and HS-106 synergistic effect on apoptosis in Lapatinib resistant BT474. Lapatinib resistant BT474 cells were treated with different concentrations of HS-106 with 10µM Lapatinib for 24 hours, fixed, labeled with fluorescent Annexin V and stained with Sytox red viability dye. HS-106 treatment was found to increase the apoptotic cells population more than the Lapatinib treatment alone.

5.2 HS-106 inhibits HER2 expression

To probe the mechanism by which HS-106 sensitizes resistant cell lines to Lapatinib, we assessed the effect of HS-106 and Lapatinib combination on HER2 surface expression in resistant cells by fluorescence microscopy. Lapatinib resistant BT474 cells were treated with HS-106 and Lapatinib for 24 hours then cells were fixed without
permeabilization and stained with anti-HER2 antibody and Alexa flour labeled secondary antibody (Figure 25).

**Figure 25**: HS-106 inhibits HER2 expression in Lapatinib resistant BT474 cells.

HS-106 treatment did not show a clear reduction in surface HER2 expression but an overall decrease of HER2 expression. To confirm HS-106 inhibition of HER2 expression, we treated Lapatinib resistant cell lines with increasing concentrations of HS-106 for 48 hours and determined HER2 and HER3 protein levels by western blot (Figure 26).
HS-106 inhibits the expression of both HER2 and HER3 in Lapatinib resistant cell lines. HS-106 was able to reduce both HER2 and HER3 levels in a dose dependent manner in Lapatinib resistant cell lines, suggesting a more general mechanism by which FASN is down regulating the expression of ErbB receptors.

5.3 Discussion

HS-106 activity in the Lapatinib resistant cell lines is consistent with the reports showing that FASN inhibition can induce apoptosis in resistant breast cancer cell lines (Puig et al., 2011; Vazquez-Martin et al., 2007). The limited amount of data that we have provides a hint to the mechanism responsible for the sensitization of resistant cell lines to Lapatinib. Based on our data, HS-106 may re-sensitize the resistant cells by decreasing the expression levels of HER2, which reduces the number of HER2 moles per each mole of Lapatinib, leading to an increase in Lapatinib efficacy. Another possible mechanism is HS-106 reduction of HER3 levels, which is a favored dimerization partner of HER2 in the event of both HER2 and EGFR kinase domain inhibition (Sergina et al., 2007).
We can only suggest possible mechanisms that explain how HS-106 or the inhibition FASN activity reduces the expression of HER2 and HER3. In the previous chapter, we discussed the probability that HS-106 anticancer activity is conducted through the disruption of lipid rafts. This disruption in lipid rafts may explain the decrease in HER2 and HER3 levels upon HS-106 treatment through the mislocalization of the receptors to the cell surface. This proposed mechanism is also consistent with other reports in the literature (Menendez et al., 2005; Ventura et al., 2015).

Another possible mechanism that can explain HS-106 activity is the possible dependence of ErbB receptors on palmitoylation or other fatty acid derived modification for activation or heterodimerization. It is reported that some prostate cancer cell lines has EGFR populations that are dependent on palmitoylation for dimerization and activation and inhibiting FASN activity would reduce these EGFR populations levels and activity (Bollu et al., 2015).
6. HS-106 shows tolerability and anti-cancer activity in vivo

Before testing HS-106 activity in vivo, we tried to determine the maximum tolerability dose (MTD) and the bioavailability of the molecule by performing IP route Pharmacokinetic study.

6.1 HS-106 is bioavailable and well tolerated in mice

We performed an acute toxicity study where FVB/J mice received 5, 20 or 80mg/kg of HS-106 via intraperitoneal injection (IP) on days 1 and 3, and blood was collected on day 4 where a complete blood workout was done.

HS-106 was toxic at 80mg/kg, but at 5 to 20 mg/kg was well tolerated with no adverse effects on white blood cell counts, hemoglobin levels, kidney, or liver functions (Figure 27).
Figure 27: FVB/J mice were treated with two doses of HS-106 for a week. After that, the mice were sacrificed and blood was collected. The samples were assayed for blood cell count (A, B and C), electrolytes (D, E and F), liver functions (G, H and I), kidney functions (J, K, and L), hemoglobin (M) and packed cell volume (N).

To test for the long term effects of HS-106, mice received biweekly IP injections of 5, 10, or 15 mg/kg HS-106 for eight weeks. None of these doses induced any signs of toxicity, stress or any significant change in mice weight (Figure 28).
Figure 28: HS-106 does not induce weight change in FVB/J mice. Mice were assessed weekly, treated BIW with an IP injection of different concentrations of HS-106 made in 1:1 DMSO/saline.

Next, we carried out a pharmacokinetic (PK) study to determine the uptake and biodistribution of HS-106 in MMTV-Neu mice. Tumor-bearing mice were IP injected with a single dose of 15mg/kg HS-106 after that, tissues and plasma were collected at different time points. HS-106 was extracted by acetonitrile from tissues homogenate which were spiked with the analog HS-247 as an internal standard (Figure 29).

![Structure of HS-247](image)

**Figure 29: Structure of HS-247**

HS-106 concentration was determined by LC-MS and using a standard curve of HS-106 and HS-247.
Figure 30: Pharmacokinetics of HS-106. (A) After one IP dose of 15mg/kg of HS-106, FVB/J mice were sacrificed at different time points and tissues was collected and assayed for HS-106 concentration by LC/MS.

The study showed that HS-106 appears rapidly in the plasma within 5 minutes of the IP injection and is cleared rapidly (T1/2 = 9.81±0.02 min n=3) (Figure 30). Similar uptake and clearance was also observed in liver and kidney (liver T1/2 = 9.84±0.09 min, n=3; kidney T1/2 = 9.90±0.01 min, n=3). Although the MS analysis focused primarily on the parent compound (amu 339Da), examination of the entire liquid chromatography profile following drug extraction of the tissues did not reveal any obvious HS-106 metabolites.
These findings suggest that HS-106 is rapidly cleared through the kidney and liver in its parent ion state.

Table 2: Pharmacokinetics of HS-106 in MMTV Neu mice.

<table>
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<tr>
<th>Tissue</th>
<th>$C_{\text{max}} \pm \text{SEM}$ (nmol/ml or nmol/g)</th>
<th>$T_{\text{max}}$ (min)</th>
<th>$T_{1/2}$ (min) $\pm$ SEM</th>
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<td>9.81 ± 0.02</td>
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<td>liver</td>
<td>16.59 ± 2.83</td>
<td>5</td>
<td>9.84 ± 0.09</td>
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<tr>
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<td>60</td>
<td>65.71 ± 0.32</td>
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<tr>
<td>Kidney</td>
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<td>5</td>
<td>9.90 ± 0.01</td>
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<tr>
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<td>15.22 ± 2.17</td>
<td>5</td>
<td>10.21 ± 0.30</td>
</tr>
<tr>
<td>Spleen</td>
<td>41.35 ± 3.37</td>
<td>5</td>
<td>8.89 ± 0.01</td>
</tr>
</tbody>
</table>

6.2 HS-106 increase survival and have potent anti-tumor activity in MMTV Neu mice when combined with Carboplatin

Having determined that HS-106 was well tolerated in mice, we next evaluated its efficacy on tumor progression in the Mouse Mammary Tumor Virus (MMTV)-Neu model of HER2+ breast cancer (Muller et al., 1988) (Jackson Labs Strain 002376). Cohorts of MMTV Neu mice were treated with a biweekly IP injection of 15 mg/kg of HS-106 (Figure 31).
Figure 31: HS-106 activity in MMTV-Neu mice. Mice were assessed weekly, treated BIW with an IP injection of different concentrations of HS-106 made in 1:1 DMSO/saline. (A, B) combination of HS-106 and Carboplatin significantly reduce tumor volume (t-test, P value=0.014). (C) HS-106 increases the median survival of the mice from 29 to 63 days with a log-rank P value of 0.049.

When administrated alone, HS-106 reduced tumor volume compared with vehicle treated animals (day 21 HS-106 treatment volume 436± 218mm n=7 SDM, control volume 628 ± 381mm n=9, p= 0.85). Significantly, HS-106 also increased the median survival of the MMTV Neu mice to 63 days (p=0.049) compared with vehicle alone treated animals (Figure 31C). Importantly, MS analysis of tumor tissue verified HS-106
uptake and also showed a significantly longer elimination time (T1/2=65.71±0.32 min n=3) than all other tissues tested. The long duration of treatment in our studies suggest the dosing frequency of HS-106 can be greatly increased to achieve greater effects on survival and tumor volume.

More dramatic acute tumor responses were observed when HS-106 was combined with 50 mg/kg of the platinum-based chemotherapeutic agent Carboplatin administered weekly. Here, 88% of tumors achieved an objective response rate of stable disease or better compared to Carboplatin only at 25%, fisher’s exact, p-value 0.01. This response was not durable however as there was no long term benefit of the combination therapy at this dosing regimen (Figure 31C). As often seen in the clinic, tumors that are responsive initially will develop resistance which is likely the case here. These findings are consistent with the actions of two compounds acting independently of one another; in which, one anti-neoplastic develops resistance while the other maybe unaffected. Importantly, carboplatin is a front line chemotherapeutic agent for the treatment of breast cancer. Similar in action to cisplatin (Knox et al., 1986), carboplatin stops tumor progression by binding to DNA and inducing a DNA damage response that leads to halt proliferation and activation of apoptosis (Chu, 1994). Although Carboplatin is less toxic than the Cisplatin (Harland et al., 1984), toxicity is still a major issue where the drug dose is determined based on the target area under the curve (AUC) and evaluated drug
clearance (Etienne et al., 2003), and in most cases is administered once every 4 weeks (Martin et al., 1992).

6.3 HS-106 has potent anti-tumor activity in C3Tag mice when combined with Carboplatin without any increase in survival

Due to the efficacy of HS-106 when combined with Carboplatin in the MMTV-Neu model, we tested a similar combination in the C3Tag mouse model of triple negative breast cancer (TNBC). Unlike HER2+ or ER+ breast cancers, TNBC does not have any molecularly targeted drugs and platinum-based compounds are the most used chemotherapeutics for treatment. The combination of HS-106 and carboplatin was able to significantly reduce tumors volume in the C3Tag model (Figure 32).
Figure 32: HS-106 activity in C3Tag triple negative mice. Mice were assessed weekly, treated BIW with an IP injection of different concentrations of HS-106 made in 1:1 DMSO/saline. (A,B and C) combination of HS-106 and Carboplatin significantly reduce tumor volume (t-test, P value=0.00221).

This reduction in tumor volume in HS-106 and Carboplatin combination treatment was not translated into any increase in mice survival (Figure 33).
Figure 33: HS-106 effect on C3Tag mice survival. HS-106 does not have any effect on the survival of C3Tag mice.

6.3 Discussion

In contrast to other FASN inhibitors, HS-106 is well tolerated in mice and does not induce any overt weight loss or any change in feeding behavior. Even on a conservative twice weekly dosing regimen, HS-106 showed a profound effect on the median survival of MMTV Neu mice. Moreover, combining HS-106 with Carboplatin synergistically reduced tumor volumes and impacted survival over the first 40 days of combination treatment. Although, overall survival was not extended beyond HS-106 alone, the dramatic early response to the combination has clinical relevance. Normally, Carboplatin treatment is restricted to 21 days in patients due to its toxicity and tendency to develop resistant tumors when used over the longer term. Our pharmacokinetic study shows that there is a room to increase HS-106 dosing to improve its performance in vivo.
The compound is rapidly cleared from plasma and tissues which indicates that it’s possible to increase the dosing schedule from twice weekly to at least a daily regimen. HS-106 may therefore enable significant reduction of the Carboplatin dose. This may increase the combined drugs’ efficacy while reducing the toxicity of the latter compound. In the C3Tag mouse model, HS-106 was not able to induce significant change in tumor volume or mice survival. C3Tag mouse model is suggested to have a frequent K-RAS amplification (UNC Mouse Phase 1 Unit, unpublished data). This piece of information is important because it is reported that K-RAS driven cancer cells are likely to be resistant to FASN inhibition and they can be sensitized only when the activity of PI3K and MEK is inhibited (Yellen and Foster, 2014). In summary, several approaches can be used in the future to improve HS-106 efficacy in vivo including: changing the dosing schedule or using different drug combinations.
7. Conclusion remarks and future directions

FASN presents an attractive target for the treatment of cancer and currently there is only one molecule going through phase I clinical trials. Our interest in developing a FASN inhibitor stems from the presence of a very limited number of effective and selective FASN inhibitors and the absence of molecules targeting the co-factors binding sites. Our FLECS screen was able to successfully identify several hits that elute FASN from blue Sepharose resin. Although the initial hit rate was high (7.3%), it did not generate a problem for us due to the small number of molecules in our library (3379 molecule).

The thiophenopyrimididine scaffold hit from our screen (HS-106), was chosen as a lead molecule based on its high activity in inhibiting fatty acid synthesis in the cell based assays. The ability of purine containing nucleotides to elute FASN of the blue Sepharose resin suggests that FASN could be binding through the ketoacyl reductase, enoyl reductase or the malonyl/acetyl transferase domains. All these domains use one of the purine-containing co-factors in the form of NADPH, acetyl-CoA or malonyl-CoA. HS-106 ability to elute FASN is possibly through binding to one of the previously mentioned domains (Figure 34). Due to the lack of high resolution crystal structure for any of these domains, we were not able to perform any docking studies to narrow down HS-106 putative binding sites.
To pursue which domain or partial enzymatic activity is targeted by HS-106, it is possible to assay the enzyme activity for each domain using substrates similar to the fatty acids synthesis intermediates. The disadvantage of such method is that these
substrates are not the native ones for the domains activity which make them less reliable in emulating the intermediate reactions that occur during fatty acid synthesis. Another approach that can be used is the cloning of a GFP fusion protein for each domain and eluting with HS-106 of the blue Sepharose resin. The only drawback of this approach is the possible loss of binding ability of these domains due to the absence of the other domains that make them fold properly.

We tested HS-106 activity in several breast cancer cell lines and the molecule showed more toxicity in the aggressive breast cancer cell lines. We tried to delineate the mechanism by which HS-106 induces apoptosis in the HER2+ breast cancer cell lines. This was done by performed several rescue experiments and detection of changes in lipids homeostasis induced by HS-106. We found that there are two major events that take place upon the inhibition of FASN activity by HS-106 that leads to second set of events and ends with the induction of apoptosis. First, FASN inhibition leads to the reduction in phospholipids synthesis from endogenous fatty acids and induce an increase in the uptake of exogenous fatty acids. These fatty acids are channeled for the synthesis of neutral lipids instead of phospholipids due to the glucose being directed to glycerol synthesis leading to more diacylglycerols triglycerides formation. The second event is the inhibition of fatty acids oxidation by the accumulation of malonyl-CoA which leads to an increase in ceramides levels. Both events prime the disruption of lipid
rafts by decrease phospholipids synthesis and the increase in ceramides levels leading to
the induction of apoptosis (Figure 35).

Figure 35: Proposed model for HS-106 induced apoptosis.

This proposed mechanism suggests that inhibition of FASN would be deleterious
for the growth of cancer cell lines that depends on survival and proliferation signals
conducted by cell surface receptors. This mechanism can be further qualified by directly
evaluating the effect of HS-106 on lipid rafts and receptors localization. Moreover,
testing HS-106 effect on lipidome in different media conditions such as different glucose,
glutamine and fatty acids concentration would give more information about the
pathways affected by the blocking of fatty acid synthesis. Also using isotope tracers such as \(^{11}\)C Acetate would make it easier to track the source of each identified lipid species.

Our HS-106 efficacy studies in Lapatinib resistant cell lines showed that HS-106 synergize Lapatinib activity. Also, HS-106 was found to decrease the expression levels of both HER2 and HER3. Although Lapatinib is close to be replaced by the new generation of tyrosine kinase receptor inhibitors, these molecules are most likely will have similar resistant mechanism which means that the inhibition of FASN will still be a valuable strategy to combat drug resistance.

HS-106 showed good tolerability in mice with a dose of 15mg/kg twice weekly. On its own, HS-106 was not able to significantly reduce tumor size in the MMTV-Neu or C3Tag mouse models. When combined with Carboplatin, significant reduction in tumor volume was observed in both mice models. The mice survival on the other hand, was not increased in the combination more than the Carboplatin alone. However, HS-106 treatment doubled the median survival of MMTV-Neu mice. The low efficacy of HS-106 monotherapy is likely due to the rapid elimination of the drug from the mice system as shown by our PK study results. This suggests that more frequent dosing of HS-106 would probably enhance the drug efficacy.

The feasibility of HS-106 synthesis by one efficient reaction suggests that many analogs can be rapidly synthesized (Figure 36).
Proposed synthetic strategy for HS-106 analogs. HS-106 can be synthesized by reacting 4-Chloro-5, 6-dimethylthieno [2, 3-d] pyrimidine (which form region A) with 1-benzyl-3-aminopyrididine (region B). A variety of starting materials for each region can be purchased and used for the synthesis of many HS-106 analogs.

These generated analogs can be tested for SAR using the blue Sepharose screening method. Although such a method would not be sensitive enough to detect the small changes in activity between analogs, it would provide an efficient filter to eliminate inactive analogs or highly unselective ones. After that, a secondary screen can be developed based on monitoring the incorporation of $^3$H acetate into lipids to find molecules with the highest cellular activity and eliminate weak or non-cell permeable
ones. Analogs with the highest activity would then be tested in the direct FASN activity assay which monitors $^{14}$C malonyl-CoA incorporation into palmitate.

Such a lead optimization strategy would help in the identification of analogs that has better activity than HS-106 that can advance into more in vivo studies.
Appendix A

Synthesis of HS-106

HS-106 was originally obtained from Enamine Ltd. (www.enamine.com, T5790201) but is no longer available from them. (N-(1-benzylpyrrolidin-3-yl)-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine benzenesulfonate) HS-106 4-Chloro-5,6-dimethylthieno[2,3-d]pyrimidine (1.02 g, 5.13 mmol) and 1-benzyl-3-aminopyrrolidine (1.09 g, 6.16 mmol) were combined and treated with Hunig’s base (1.33 g, 10.3 mmol) and ethanol (4 mL). The mixture was heated to 100°C for 2 hours. The mixture was concentrated to an oil and chromatographed [silica gel 3.5 x 25 cm, ethyl acetate (250mL), followed by 9/1 : ethyl acetate/methanol (400 mL)]. The product was dissolved in ethyl acetate and treated with toluene sulfonic acid (1 g) in ethyl acetate and stirred vigorously. The crystalline solid was filtered off and aid dried to give N-(1-benzylpyrrolidin-3-yl)-5, 6-dimethylthieno[2, 3-d] pyrimidin-4-amine benzenesulfonate (HS-106, 1.8 g, 70%) as a white powder. TLC Rf = 0.21 in ethyl acetate, Rf = 0.39 in 9/1 :
CH2Cl2/methanol; LC/MS m/z = 339.2; 1H NMR (CD3OD) δ 8.29 (s, 1H), 7.68 (d, J = 7.8 Hz, 2H, TsOH), 7.53 (br m, 2H), 7.42 (m, 4H), 7.15 (d, J = 7.8 Hz, 2H, TsOH), 4.99 (m, 1H), 4.50 (d, J = 13 Hz, 1H), 4.30 (d, J = 13 Hz, 1H), 3.89 (m, 1H), 3.55 (m, 1H), 3.48 (dd, J = 4.3, 12 Hz, 1H), 3.27 (M, 1H), 2.68 (m, 1 H), 2.41 (s, 3H), 2.40 (s, 3H), 2.3-2.5 (m, 2H), 2.35 (s, 3H, TsOH).
Appendix B

Synthesis of HS-106 enantiomers

(R)-N-(1-benzylpyrrolidin-3-yl)-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine HS-79. 4-Chloro-5,6-dimethylthieno[2,3-d]pyrimidine (100 mg, 503 μmol) and (R)-(−)-1-benzyl-3-aminopyrrolidine (Aldrich, 89 mg, 503 μmol) were combined, treated with Hunig’s base (130 mg, 1 mmol) and ethanol (700 μL) and heated to 100 °C for 2 h. The reaction mixture was allowed to cool, diluted with DMSO (500 μL) and purified by prep HPLC (5 to 100% methanol with 0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give the product, a formate salt (190 mg, 98%) as a clear glass. LC/MS showed pure product (m/z = 339.3, [M+1]+) to be identical to the racemic commercial sample.

(S)-N-(1-benzylpyrrolidin-3-yl)-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine HS-80. 4-Chloro-5,6-dimethylthieno[2,3-d]pyrimidine (100 mg, 503 μmol) and (S)-(−)-1-benzyl-3-aminopyrrolidine (Aldrich, 89 mg, 503 μmol) were combined, treated with Hunig’s base (130 mg, 1 mmol) and ethanol (700 μL) and heated to 100 °C for 2 h. The reaction mixture was allowed to cool, diluted with DMSO (500 μL) and purified by prep HPLC (5 to 100% methanol with 0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give the product, a formate salt (150 mg, 78%) as a clear glass. LC/MS showed pure product (m/z = 339.3, [M+1]+) to be identical to the racemic commercial sample.

5,6-dimethyl-N-(pyrrolidin-3-yl)thieno[2,3-d]pyrimidin-4-amine, hydrochloride HS-102. 4-Chloro-5,6-dimethylthieno[2,3-d]pyrimidine (100 mg, 503 μmol) and 1-BOC-3-
aminopyrididine (103 mg, 554 μmol) were combined and treated with Hunig’s base (130 mg, 1 mmol) and ethanol (700 μL) and heated to 70 °C for 18 h. The reaction mixture was concentrated to an oil and chromatographed (silica gel, 9/1 : CH₂Cl₂/MeOH ) to give the intermediate as a glass. The glass was dissolved in methylene chloride (~4 mL) and treated with TFA (~1 mL). After about 1 h, the reaction mixture was concentrated, dissolved in DMSO (~1 mL) and purified by prep HPLC (0 to 100% methanol, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give the product (~136 mg) as an oil. The oil was dissolved in ethanol and treated with 60 uL of 12 N HCl, which caused a lot of solid formation. The mixture was heated to reflux, allowed to cool, and filtered off and air dried to give product (90 mg, 63%) as a white powder. LC/MS showed a pure product with an m/z = 249.1, [M+1]⁺.
## Appendix C

Lipidomics ESI+ data (p<0.001, > 2 fold difference between treatments)

<table>
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<th>Compound</th>
<th>m/z</th>
<th>Retention time (min)</th>
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<th>SD DMSO</th>
<th>CV DMSO</th>
<th>average HS-106</th>
<th>SD HS-106</th>
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## Appendix D

**Lipidomics ESI+ data (p<0.001, all identified)**

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## Appendix E

Lipidomics ESI- data (p<0.001,>2 fold difference between treatments)

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## Appendix F

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References


acid synthase null mutants and most of the heterozygotes die in utero. Proceedings of the National Academy of Sciences of the United States of America 100, 6358-6363.


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