TARGETING FABP5 WITH SMALL-MOLECULE INHIBITORS AND ASSESSING THE IMPACT ON RETINOIC ACID (RA)-RESISTANT CANCERS

by

ELIZABETH MEYERS

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Dissertation Advisor: Dr. Gregory P. Tochtrop

Department of Chemistry

CASE WESTERN RESERVE UNIVERSITY

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CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the dissertation of

Elizabeth Meyers

candidate for the degree of Doctor of Philosophy*.

Committee Chair

Dr. Fu-Sen Liang

Committee Member

Dr. Gregory Tochtrop

Committee Member

Dr. Blanton Tolbert

Committee Member

Dr. Anna C. Samia

Committee Member

Dr. Liraz Levi

Date of Defense

December 3, 2019

*We also certify that written approval has been obtained

for any proprietary material contained therein.

For Tom, Charlotte, and Amelia.

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LIST OF SYMBOLS AND ABBREVIATIONS

Å	Angstrom
^0C	Degrees Celsius
μg	Microgram
μM	Micromolar
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
ADH	Alcohol dehydrogenase
BCM01	Beta-carotene oxygenase 1
$CDCl_3$	Deuterated chloroform
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CRABP2	Cellular Retinoic acid binding protein 2
DBD	DNA binding domain
DCM	Dichloromethane
DGAT1	Diacylglycerol acyltransferase1
DHA	Docosahexaenoic acid
DME	Dimethoxyethane
DMEM	Dulbecco's modified Eagle meida
DMF	Dimethylformamide
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FABP5	Fatty-acid binding protein 5

hFABP5	Human fatty-acid binding protein 5
FBS	Fetal bovine serum
g	Grams
HER2	Human epidermal growth factor receptor 2
HuR	Human antigen R protein
hr	Hours
HRP	Horseradish peroxidase
iLBP	Intracellular bindig protein
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IU	International unit
KLF2	Kruppel-like factor 2
LB	Lysogeny broth
LBD	Ligand-binding domain
LCFA	Long-chain fatty acids
LRAT	Lecithin:retinol acyltransferase
MeOD	Deuterated methanol
mg	Milligram
$MgCl_2$	Magnesium chloride
MHz	Megahertz
min	minutes
mL	Milliliter
mM	Millimolar
mmol	Millimoles

mRNA	Messenger ribonucleic acid
n-BuLi	n-Butyllithium
NaCl	sodium chloride
N-CoR	nuclear receptor corepressor
NLS	Nuclear localization sequence
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Nuclear receptors
NURR1	Nuclear receptor related 1 protein
O.D.	Optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferation-activated receptor
PPARα	Peroxisome proliferation- activated receptor α
PPARγ	Peroxisome proliferation- activated receptor γ
PPARδ	Peroxisome proliferation-associated receptor δ
PPRE	Peroxisome proliferator response element
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARα	Retinoic acid receptor alpha
RARβ	Retinoic acid receptor beta
RARγ	Retinoic acid receptor gamma

RARE	Retinoic acid response element
RBP2	Cellular retinol binding protein 2
RBP4	Cellular retinol binding protein 4
RDH	Retinol dehydrogenase
RE	Response elements
REH	Retinyl ester hydrolase
qRT-PCR	Quantitative real-time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROR	Retinoid orphan receptor
RPM	Rotations per minute
RT	Room temperature
RXR	Retinoid X receptor
RXRα	Retinoid X receptor α
RXRβ	Retinoid X receptor β
RXRγ	Retinoid X receptor γ
SAR	Structure-activity relationship
SMRT	silencing mediator for RARs and TRs
SDS	Sodium dodecyl sulfate
STRA6	Stimulated by retinoic acid 6
TBST	Tris-buffered saline with Tween 20
THF	Tetrahydrofuran
TNFα	Tumor necrosis factor α

TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
XRD	X-ray diffraction

Targeting FABP5 with Small-Molecule Inhibitors and Assessing the Impact on Retinoic Acid (RA)-Resistant Cancers

Abstract

by

ELIZABETH MEYERS

While atRA canonically enacts gene transcription through the CRABP2/RAR signaling pathway to induce anti-carcinogenic effects, it is also able to act through the FABP5/PPARδ signaling pathway to induce pro-proliferative effects. This dual-activation by atRA leads to a dual response to atRA treatment of certain cancers. The central goal of this work is to target an inhibitor to FABP5 so that in those instances where atRA induces tumor growth, the hormone will be re-directed to the CRABP2/RAR anti-proliferative signaling pathway instead. After screening several candidate inhibitors, it was found that two compounds, 5, or AM404, and 6, a multi-ring amine compound, are able to effectively inhibit FABP5 and reduce proliferation and PPARδ signaling in the triple-negative breast cancer cell line MDA-MB-231.

The structure-activity relationship (SAR) of compound 6 and FABP5 is quite complicated and ultimately unclear. It was found that the endo isomer of the compound is most effective at inhibition, but the required chirality on specific carbons remains murky. In the process of exploring the SAR, it was found that a mixture of compound 6 isomers may improve inhibitor performance. Furthermore, this mixture is able to activate PPARα, which may provide a new avenue for cancer treatment with this compound via manipulation of fatty acid metabolism.

CHAPTER 1. INTRODUCTION

1.1 VITAMIN A: METABOLISM AND CELLULAR EFFECTS

Vitamin A is essential to healthy embryonic development, growth, immunity, reproduction and vision.[1–5] Early rodent studies found that vitamin A deficiency induces hyperplasia and squamous metaplasia and can be reversed when retinoids are supplemented back into the diet.[6,7] In humans, vitamin A deficiency is a major cause of blindness, attenuated growth, and death for pregnant women and young children, particularly in developing countries.[8–11] Early intervention via vitamin A supplementation to pregnant women and infants can reverse vision loss and increase innate immune response in infants and young children.[12,13]

Retinoids and the pro-vitamin A precursors carotenoids are not synthesized *de novo* by vertebrates and are acquired from the diet. Dietary retinoids primarily derived from plant sources as β-carotene, the most abundant carotenoid, that is a terpenoid composed of two retinyl groups (Figure 1.1). Vitamin A may also be obtained from animal sources as retinol and retinyl-ester derivatives, usually as retinyl-palmitate.[14,15] Uptake and further metabolism of dietary retinoids and carotenoids occurs in the intestinal enterocytes. Dietary retinyl-esters must first be hydrolyzed to retinol within the intestinal lumen or at the brush border. Once absorbed, retinol binds cellular retinol binding protein 2 (RBP2) that delivers the nutrient to one of the gut enzymes lecithin: retinol acyltransferase (LRAT) or diacylglycerol acyltransferase 1 (DGAT1) for re-esterification. The newly formed retinyl-esters are then packed in nascent chylomicrons together with other lipids such as triglycerides and cholesteryl esters, and secreted into the lymphatic system and into the circulation for uptake into peripherial tissues. Dietary

carotenoids like β-carotene are taken into intestinal enterocytes and immediately converted into retinaldehyde and bound by RBP2. Following conversion of the retinaldehyde to retinol the molecule is also esterified and packed into chylomicrons. Liver hepatocytes are able to internalize the retinyl ester-containing chylomicron remnant, absorb the retinyl-esters and re-hydrolyze them into retinol. The retinol may then be converted back into retinyl-ester for storage in liver stellate cells or sent out into circulation bound to retinol binding protein 4 (RBP4).[15]

Cellular uptake of retinol in peripheral tissues is driven by the membrane-bound protein stimulated by retinoic acid 6 (STRA6). STRA6 recognizes RBP4-bound retinol and initiates its release to RBPs in the cell.[16] The fate of RBP-bound retinol varies widely with the specific type and function of the target cell.[17] The major active metabolites of retinol are 11-*cis* retinal, 9-cis-retinoic acid, and all-trans retinoic acid. In the eyes, the retinal pigment epithelium in rod and cone cells converts absorbed retinol into 11-cis-retinal, where it goes on to bind opsin and initiate the visual cycle.[5] Outside of the visual cycle, cellular retinol may be converted into 9-*cis*-retinoic acid or all-*trans* retinoic acid (atRA). atRA and 9-*cis*-retinoic acid are active in initiating gene transcription and play a significant role in a wide variety of biological processes, including embryonic development, gene transcription, and cell cycle regulation.[18–20]

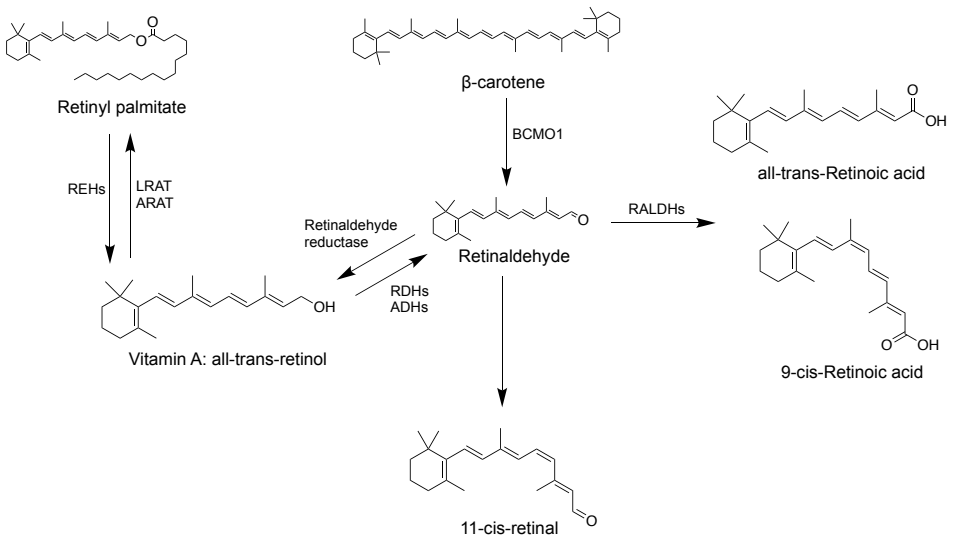

Figure 1.1 Metabolic fates of Vitamin A. Vitamin A is primarily obtained from the diet as β-carotene from plant sources and from some animal sources in the form of retinyl esters, most commonly retinyl palmitate. Both pro-vitamin A compounds may be converted into vitamin A, or all-*trans* retinol, via retinyl ester hydrolases (REHs) or retinaldehyde reductase. Vitamin A may then be stored or converted into retinaldehyde via retinol or alcohol dehydrogenases (RDH; ADH), depending on the needs of the cell. Retinaldehyde may then be converted into 11-*cis*-retinal as part of the visual cycle or converted into all-*trans* retinoic acid and 9-*cis* retinoic acid via retinaldehyde dehydrogenase (RALDH) to enact gene transcription.

Retinoic acid regulates gene transcription by activating three members of the nuclear receptor family of ligand-activated transcription factors: retinoic acid receptor (RAR), retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor δ (PPARδ). atRA regulates classical RA receptors RARα, RARβ and RARγ and the non-canonical RA receptor PPARδ while 9-*cis* RA activated the transcriptional activity of RXR receptors RXRα, RXRβ, and RXRγ.[21] Understanding the mechanisms of these RA-activated nuclear receptors is key to understanding the transcriptional activities of atRA.

1.2 NUCLEAR RECEPTORS

Nuclear receptors (NR) are a class of receptor proteins that, unlike membrane-bound signaling proteins, bind directly to DNA and respond to intracellular signals by initiating transcription of specific target genes.[22] Nuclear receptors are all composed of five general structural domains: the DNA-binding domain (DBD), ligand-binding domain (LBD), hinge region, and N-terminal and C-terminal domains.[23–25] Among these structural domains, DBD and LBD display high sequence and structural homology among different NR family members.[23] The DBD is composed of two zinc-fingers that bind to specific DNA sequences called response elements. This domain also mediates the homo- or heterodimerization of that specific nuclear receptor.[23] The LBD is likewise responsible for binding the respective ligand of the NR and acts as a molecular switch for the protein, converting it to a transcriptionally active state.[23,24] The remaining structural domains are less well-conserved among NR family members and are responsible for connection, transactivation of transcription and regulation of ligand binding.[23]

There are 48 known human nuclear receptors that can be divided into 6 subfamilies based on the sequence homology of their DNA-binding and ligand-binding domains: thyroid hormone receptor-like, retinoid x receptor-like, estrogen receptor-like, nerve growth factor IB-like, steroidogenic factor-like and germ cell factor-like.[26–28] The endogenous ligands for these subfamilies are highly varied but can generally be characterized as lipophilic molecules including hormones such as estrogen and androgen, vitamins A and D, and fatty acids.[29–34] In addition to these subfamilies, some NR, such as retinoid orphan receptor (ROR) and nuclear receptor related 1 protein (NURR1), lack any known or generally agreed upon ligands and can be classified as orphan receptors.

Orphan receptors are not functionally related and have little to no sequence or structural homology.[35,36]

1.2.1 Type II Nuclear Receptors

The nuclear receptor superfamily can be further categorized by mechanism of action. Types I and III bind either inverted or direct response elements (RE), respectively, as a homodimer and reside in the cytosol while type IV NR can bind RE as monomers or dimers.[37] Of particular interest are type II NR which almost exclusively bind their respective RE as a retinoid X receptor (RXR) hetero-dimer and, in contrast to Type I, reside exclusively in the nucleus.[24,38,39] Most type II NR are complexed with a corepressor protein such as silencing mediator for RARs and TRs (SMRT) or nuclear receptor corepressor (N-CoR) (Figure 1.2).[39] When a ligand enters the nucleus and binds its target receptor, the corepressor is displaced and replaced with a coactivator protein, such as TIF1, ERAP160, or RIP140, that then recruits RNA polymerase to the NR binding site.[39] Once the coactivator-polymerase complex is bound, the RNA polymerase will begin transcribing the NR target gene(s), resulting in changes in protein expression that modulate cell function.[20,38]

One of the chief distinguishing features of type II nuclear receptors is the almost exclusive heterodimerization with retinoid X receptor (RXR). RXR is categorized as an orphan nuclear receptor due to the controversy surrounding its endogenous ligands, but in recent years, both 9-cis RA and the poly-unsaturated fatty acid docosahexaenoic acid (DHA) have been discovered bound to RXR in tissues.[40,41] There are three isoforms of RXR: RXRα, RXRβ, and RXRγ. The expression levels of these isoforms vary by tissue

type and cell differentiation, with the alpha isoform primarily expressed in the epidermis, liver, kidney and intestine while the gamma isoform is expressed in brain, muscle and at low levels in adipose tissue. RXRβ is ubiquitously expressed.[42,43] Structurally, the RXR family has the same basic structure as other known NR, with two zinc fingers in the DBD.[42] It largely functions as a transcription factor and can be found as either a homo or heterodimer with members of the type II NR subfamily, but there is evidence for RXR participation in TR3 nuclear export in apoptotic events and inhibition of platelet aggregation.[44–47] In the larger context of atRA signaling, however, the most critical function of RXR is its heterodimerization with the type II NRs RAR and PPARδ.

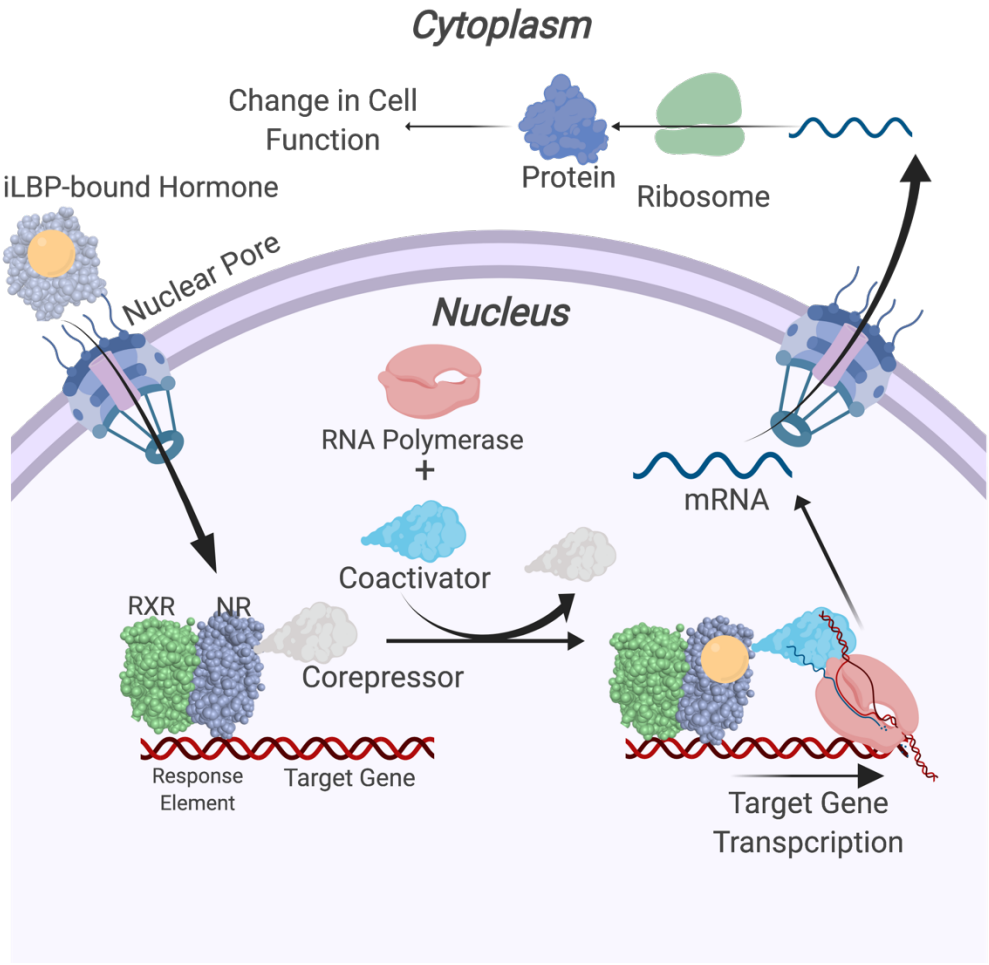

Figure 1.2. Gene transcription pathway for type II nuclear receptors. Type II nuclear receptors bind to their response elements next to target genes as an RXR heterodimer. When a signaling hormone enters the nucleus via nuclear pore, it displaces a corepressor on the NR, thus recruiting a coactivator protein coupled with RNA polymerase. Transcription of the target gene is initiated, leading to mRNA that leaves the nucleus and initiates the synthesis of proteins that go on to change cell function.

1.2.2 Retinoic Acid Receptor (RAR)

Retinoic acid receptor (RAR) is a type II NR that is activated by all-trans retinoic acid and 9-cis-retinoic acid.[40] There are three known RAR nuclear receptors, RARα, RARβ, and RARγ, each encoded by a different gene.[48–50] Isoforms that differ in N-terminal or 5'-untranslated region sequences exist for each gene.[24,51] Structurally, all

three known variants of RAR have the same basic components of other nuclear receptors, with the DNA-binding domain highly conserved between α, β, and γ forms. The ligand-binding domain is moderately conserved with sequence variations dictating the subtle differences in ligand-binding behavior.[20] Like other type II nuclear receptors, RARs exact transcriptional activation by binding to retinoic acid response elements (RARE) as either a homodimer or most often as a heterodimer with RXR.[20] The RAR/RXR heterodimer dictates interaction with the DNA major groove at the RARE sequence, making the dimer crucial for proper sequence recognition in transcription events.[52,53] While the genomic effects of RAR are represented by its transcriptional activation of target genes, the ligand-activated RAR/RXR heterodimer can render non-genomic effects by inducing extra-nuclear activation of kinase-cascades, thus initiating post-translational modifications that crosstalk with the genomic effects of RAR/RXR activation.[54–59]

Several retinoids are able to bind and activate the RAR/RXR heterodimer, yet the primary endogenous ligand of all forms of RAR is atRA.[20,39] While both 9-*cis*-RA and atRA bind the RAR/RXR heterodimer with affinities in the 0.2-0.7nM range, it is the interaction of atRA with RAR/RXR that renders the majority of genomic and non-genomic effects.[40,60,61] When atRA enters the nucleus, it binds to the RAR/RXR heterodimer which, once activated, may initiate the transcription of over 500 specific target genes. Several of these genes, including *Cyp26a1, Arg1, Dhrs3, Stra6,* and *Rarb,* directly regulate the storage, metabolism, and transport of atRA.[62–65] Others, including *Hoxa1* and *Cdx1* relate directly to embryonic development.[66,67] Related to this category of RA-inducible genes that regulate embryonic development are those that govern cell cycle regulation. For example, *CyclinD*, which regulates cell cycle progression during the

G1/S phase transition, is inducible in several breast cancer cell lines after co-treatment with atRA and ω-3 free fatty acids.[68] *Btg2* and *Tob1*, also regulators of the G1/S phase transition, are also directly upregulated after atRA activation of the RAR/RXR heterodimer in the MCF7 breast cancer cell line.[69] Retinoids, particularly atRA, were also found to induce apoptosis and reduce the carcinogenic character of several *in vitro* cell lines, including oral, skin, mammary, prostate, ovarian and bladder cancer.[38,70–73] Furthermore, atRA was found to prevent the formation of mammary tumors in mice and induce differentiation in HL60 cells, a cultured model of acute promyelocytic lukemia.[20,38,74] atRA can also inhibit adipogenesis in pre-adipocytes by triggering a signaling cascade that upregulates *Sox9*, a gene that blocks adipocyte differentiation.[75–77] This ability to arrest cell cycle progression and induce apoptosis make atRA and its canonical RAR signaling pathway a particularly attractive target for developing new cancer chemotherapeutics.

Among its uses in cancer treatment, perhaps the most successful is the use of atRA in treating acute promyelocytic leukemia (APL). APL is a subtype of acute myeloid leukemia and is unique in its morphology and particularly aggressive and fatal course.[78] About 95% of APL patients present a reciprocal translocation between the *RARA* gene on chromosome 17 and the promyelocytic leukemia gene on chromosome 15.[79] This mutation results in a fusion between the RXR/RARα heterodimer and its co-repressor protein, requiring a much higher dose of atRA to induce dissociation of the co-repressor and initiate differentiation in the cancerous cells.[79] This same strategy of atRA- or 13-cis RA-induced differentiation is also used to successfully treat high-risk pediatric

neuroblastoma and may provide a way forward in treating non-APL acute myeloid leukemia.[80–83]

While atRA has been successfully implemented as an effective treatment in these cancers, there is substantial evidence that the effect of atRA treatment is not uniform among cancer subtypes. Resistance to atRA treatment can develop in previously responsive APL patients and there is evidence for innate atRA resistance in several cancer subtypes, including MYCN-amplified neuroblastoma and glioblastoma.[82,84,85] When *MMTV-Neu* mice, a model of human HER2+ breast cancer, are treated with atRA, treatment with the compound induces tumor growth and significantly decreases survival of the mice.[86] atRA treatment has also induced growth in certain skin tumors, colon and breast cancers.[87–89] Strikingly, a clinical trial that sought to evaluate the chemopreventative potential of atRA on patients at risk for developing lung cancer saw a significant increase in the risk of developing lung cancer and an increase in deaths from lung cancer and cardiovascular disease in those patients treated with atRA when compared to the placebo population. This lead to termination of the clinical trial 21 months early.[90] These differential effects of atRA may be explained by the loss of RAR and other signaling machinery, impaired displacement of the corepressor in the type-II NR signaling mechanism, or defects in atRA synthesis present in the tumor.[91–94] However, another possible explanation lies in the atRA-mediated activation of another type-II NR, peroxisome proliferator-activated receptor δ (PPARδ).

1.2.3 Peroxisome proliferation-activated receptor δ (PPARδ)

Peroxisome proliferation-activated receptors (PPARs) are type II nuclear receptors with a wide variety of endogenous ligands, including fatty acids and eicosanoids.[95] Like most type II NR, the general structure of this family of proteins is made up of 13 helices and a small, 4-stranded β-sheet. These components compose a DNA-binding domain in the N-terminus which bind to peroxisome proliferator response elements (PPRE) and a ligand-binding domain in the C-terminus.[95,96] The ligand binding domain of PPARs contains an extra helix and is substantially larger than the binding pocket of other NRs, enabling a wide variety of possible ligands for the PPARs.[95]

There are three known isotypes of PPAR: PPARα, PPARδ, and PPARγ. Each isotype bears high sequence and structural similarity, with about 80% of residues conserved in the binding pocket of each isotype.[95] PPARα is expressed in tissues with high energy needs, including the liver, kidney, heart, skeletal muscle, and brown adipose tissue. There is also significant PPARα expression in the intestinal mucosa and most cell types present in the vascular system.[97] The target genes transcribed by PPARα contribute to the uptake and oxidation of fatty acids and lipoprotein metabolism, and as such its natural ligands include oxidized phospholipids, lipoprotein lipolytic products, and fatty acids and their metabolites.[95,97] There are several clinically relevant PPARα agonists, including fibrate drugs such as ciprofibrate and fenofibrate that work to lower systemic triglycerides and raise high-density lipoprotein cholesterol levels.[97,98]

PPARγ exists in two isoforms: PPARγ_1 and PPARγ_2. PPAR γ_1 is predominantly expressed in brown and white adipose tissue, the large intestine, and in macrophages. It is also found in various other tissues including skeletal muscle and pancreas. PPARγ_2 is

expressed in adipose tissue.[99] When activated, PPARγ modulates glucose and lipid metabolism, induces differentiation in adipose tissue, increases triglyceride synthesis, increases neuronal differentiation and reduces memory impairment, increases fatty acid oxidation in skeletal muscle and decreases inflammation in the intestine. Its natural agonists include n-6 and n-3 polyunsaturated fatty acids, eicosanoids, and nitrated fatty acids.[99] PPARγ expression may also be repressed via atRA activation of Kruppel-like factor 2 (KLF2).[100] A class of synthetic PPARγ agonists called thiazolidiendiones increase insulin sensitivity and are used in treating type 2 diabetes.[95,99]

Unlike the other PPAR isotypes, PPARδ is expressed in most cell types, with the highest expression levels in skin, brown adipose tissue, brain, kidney, liver, lung, and vascular tissues.[101] The exact functions of this PPAR isotype are harder to pinpoint due to the ubiquity of PPARδ expression, but its target genes participate in lipid metabolism, inflammation, atherosclerosis, differentiation, and in adipocyte differentiation in cooperation with PPARγ target genes.[95,101] Several PPARδ target genes are closely associated with insulin response, including the glucose transporter GLUT4.[102] PPARδ also promotes anti-apoptotic activities via transcriptional activation of the PDK1 gene, which activates the survival factor Akt1. This anti-apoptotic activity correlates to the increased expression of PPARδ in hyper-proliferative skin lesions and skin injuries.[103,104] While the role of PPARδ in the pro-oncogenic activities of cancer cells is still putative, there is strong evidence that the NR promotes proliferation and survival of cancer cells by decreasing the expression of the PTEN tumor suppressor and promoting the expression of survival factors, oncogenes, and angiogenic factors.[105]

Fatty acids, and in particular mono- and polyunsaturated fatty acids, are the primary PPARδ natural ligands along with some eicosanoids. There are no current drugs that specifically target PPARδ, yet several synthetic ligands, including GW0742 and L165041, act as potent agonists and ameliorate its metabolic functions.[106,107] Clinical trials for GW501516, a PPARδ agonist intended to treat metabolic and cardiovascular disease, were halted at phase II due to the increase in uterine, liver, bladder, thyroid, tongue, stomach, skin, testicular, and ovarian cancers in rats treated with this agonist.[108–110] While PPARδ shares endogenous ligands with the other PPAR isotypes, it retains one unique activating ligand: all-trans retinoic acid. When purified human PPAR α, γ, and δ were titrated with atRA, it was found that atRA binds specifically to PPARδ with a K_D of about 17nM, an affinity comparable to other natural PPAR ligands. PPARα and γ displayed only weak affinity for the ligand. Furthermore, atRA was able to promote interaction with transcriptional co-activators and induce transcriptional activation of PPARδ target genes *in vitro* and *in vivo*.[111] When keratinocytes were treated with atRA, there was significant upregulation of known PPARδ target genes. These same genes were upregulated after treatment with a synthetic PPARδ ligand but not after treatment with an RAR-specific agonist.[89] This indicates that not only is atRA a ligand for PPARδ, but when PPARδ activated by atRA, pro-proliferative and anti-apoptotic activities are promoted. This is in direct opposition to the known anti-proliferative activities of RAR after atRA activation. This paradoxical dual-activity of atRA via activation of both RAR and PPARδ likely provides an explanation for resistance or growth in response to atRA treatment in certain cancer types. However, the binding affinity (K_D) of atRA to RAR is about 0.2-0.7nM, or about 200-fold better than the binding affinity of atRA to PPARδ.[40]

This suggests that NR activation alone cannot explain this dual activity and that other factors must be at play in order to facilitate atRA-mediated activation of PPARδ.

1.3 iLBPs AND atRA SIGNALING: CRABP2 AND FABP5

The family of the intracellular lipid binding proteins (iLBP) is composed of 14 – 15 kDa monomeric soluble proteins that non-covalently bind retinoids, fatty acids, and other lipids in a type-specific manner.[112] The iLBP proteins bear remarkable sequence and structural similarity among its family members. All iLBPs are composed of ten anti-parallel β-strands forming a β-barrel structure with a helix-turn-helix ligand binding motif.[113] It was originally thought that these proteins served only to solubilize lipophilic compounds in the aqueous cytosol, but their unique ligand and target selectivity allow for precise and nuanced regulation of ligand localization to the peroxisomes, mitochondria, endoplasmic reticulum and nucleus.[113–115] The scientific interest in these proteins gained increased attention in recent years as members of the family were found to be involved in uptake, transport and storage of fatty acids in the cell, gene transcription, modulation of enzyme activities affecting lipid metabolic pathways, in signal transduction, differentiation and growth regulation, cognitive function and progression of cancer.[116–122]

iLBPs may be divided into four subfamilies based on phylogenetic analysis and ligand specificity: subfamily I, which includes the retinoid binding proteins cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs), subfamily II which contains the bile acid-binding proteins (BABPs) and liver-fatty acid-binding protein (FABP1), subfamily III which is comprised solely of intestinal-FABP (FABP2) and subfamily IV which contains the remaining 10 members of the FABP family.[113,123,124] The specific functions of iLBPs vary widely with tissue distribution and

the bound ligand, but several are known to translocate to the nucleus and interact with specific target nuclear receptors, including RAR, PPARα, PPARγ, and PPARδ.[105,125,126]

1.3.1 Cellular Retinoic Acid Binding Protein 2 (CRABP2)

Cellular retinoic acid binding protein 2 (CRABP2) is a member of iLBP subfamily I retinoid binding proteins and is highly specific for binding atRA. Like other iLBPs, CRABP2 is composed of ten anti-parallel β-sheets arranged in a β-barrel formation with a helix-turn-helix binding motif.[127] Apo-CRABP2 is either uniformly distributed throughout the cytosol or distributed to extra-nuclear subcellular organelles, depending on cell type. Upon treatment with atRA, however, CRABP2 will be redistributed and translocate to the nucleus.[128,129] Classically, proteins that are imported into the nucleus contain one or more sequences of positively charged lysines or arginines called a nuclear localization sequence (NLS). The classical NLS, first discovered in the SV-40 large T antigen, may be continuous, monopartite, or separated by a short sequence of amino acids, bipartite.[130,131] When the NLS of a nucleus targeted protein is recognized by importin α, an adapter karyopherin protein, it will bind importin β, another karyopherin protein. The nucleus targeted protein and importin α/β complex will then dock with a nuclear pore complex where nuclear uptake is facilitated by importin β.[132,133] While CRABP2 does not contain a classical NLS, three residues in the helix-loop-helix region of the protein, lysine 20, arginine 29, and lysine 30, rotate to the surface upon ligand binding to form a pocket of basic electrostatic potential. This pocket forms a novel tertiary NLS that mimics the location and structure the classic NLS found on the SV-40 large T antigen (Figure 1.3).[129] A K20A/R29A/K30A mutation of the CRABP2 NLS

hinders association of the mutant protein with importin α, ligand-induced nuclear localization and RAR transcriptional activity, thus proving strong evidence the critical role of the tertiary NLS in CRABP2 ligand-mediated activity.[129]

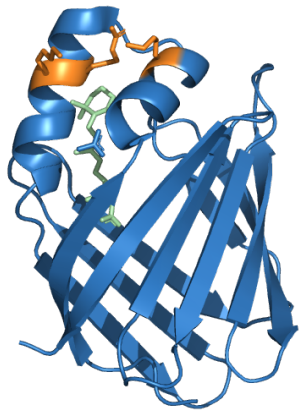

Figure 1.3 Crystal structure of holo-CRABP2 at 1.48Å resolution. Bound atRA is shown in pale green. The three residues that form the putative novel tertiary NLS, lysine 20, argenine 29, and lysine 30, are highlighted in orange on the alpha helical cap. PDB ID: 2FR3.[134]

Aside from the small rotation of the tertiary NLS residues, there is no significant structural difference between apo- and holo-CRABP2.[129,135,136] Despite this structural similarity, apo- and holo-CRABP2 are able to render anti-proliferative and oncogenic effects by two distinct mechanisms.[120] When atRA is absent, apo-CRABP2 associates with and enhances the RNA-binding affinity of the human antigen R (HuR) protein.[137] HuR is a shuttling protein that may be found in the nucleus or cytoplasm. When distributed in the cytoplasm, HuR binds to 3' untranslated regions of AU-rich mRNA, thus stabilizing the transcripts and antagonizing their degradation.[138–140] When apo-CRABP2 associates with HuR, it increases the affinity of HuR for anti-oncogenic mRNA targets, thus enhancing its ability to stabilize and promote the activity of these target

sequences.[120,137] Once atRA enters the cytoplasm, it binds CRABP2 and induces a conformational change that dissociates the HuR protein and initiates nuclear translocation.[141] When expression of HuR is eliminated, CRABP2 nuclear translocation is severely reduced, implying that HuR is also responsible for regulating expression of the genes responsible for importing CRABP2 into the nucleus.[142] In this way, CRABP2 is able to antagonize oncogenic activity even in the absence of atRA.

When atRA is present, however, CRABP2 will initiate translocation to the nucleus with proper formation of the tertiary NLS. Holo-CRABP2 will shuttle water-insoluble atRA through the cytoplasm and into the nucleus via the nuclear pore complex. While CRABP2 was previously thought to only play a role in maintaining steady state levels of atRA in the cell, the holo-protein was shown to directly upregulate the expression of RARα and RXRα when in the presence of atRA.[143,144] The rate of transfer atRA from CRABP2 to RARα is dependent on the concentration of the RAR acceptor, with a five-fold increase in RARα concentration correlating to a five-fold increase in the rate of atRA transfer. Thus, indicating that the transfer of atRA from CRABP2 to RARα depends on direct protein-protein interaction.[145] The association of CRABP2 and RARα is short-lived with atRA as the ligand and is directly responsible for overseeing the transcriptional activity of RARα.[146]

CRABP2 plays a critical role in the anti-oncogenic activity of atRA as it regulates the transcriptional activity of RAR, whose target genes include genes associated with cell cycle arrest, differentiation and apoptosis. Overexpression of CRABP2 in certain mammary carcinoma cells, sensitizes those cells to atRA-induced cell cycle arrest and apoptosis.[146,147] accordingly, growth of mammary tumors in the *MMTV-neu* breast cancer

mouse model is inhibited and survival is significantly improved in mice that overexpress CRABP2. Furthermore, as overexpressing CRABP2 sensitize the cells to atRA, exogenous application of atRA is not required to trigger the anti-oncogenic effects of CRABP2/RAR signaling path.[147] A high expression level of CRABP2 is also associated with decreased glioblastoma, astrocytic gliomas, prostate and breast cancer tumor incidence and is a marker for survival among patients.[148–151] While CRABP2 largely serves to function in an anti-oncogenic capacity by activating the transcriptional activity of RAR, there is evidence that CRABP2 promotes tumor survival and growth in lung cancer and malignant peripheral nerve sheath tumor cells.[152,153] This suggests that the anti-proliferative role of CRABP2 largely depends on cancer type. Nevertheless, the CRABP2/RAR signaling pathway is the canonical mechanism by which atRA exerts its anti-carcinogenic activity. However, some cancers are not only resistant to atRA but, paradoxically, their growth is enhanced upon treatment with the compound. This pro-carcinogenic behavior of atRA is explained by the finding that, in addition to signaling through CRABP2/RAR pathway, atRA also binds the iLPB fatty acid binding protein 5 (FABP5) and induces transcription by PPARδ.[89]

1.3.2 Fatty Acid Binding Protein 5 (FABP5)

Fatty acid binding proteins (FABPs) are a family of transport proteins with a diverse and emerging array of functions and may be categorized as subfamily II, III or IV iLBPs. While there are twelve known members of the protein family, they all are quite small at about 15kDa and bear high sequence and structure homology.[114,154] Like CRABP2, FABPs consist of a water-filled binding pocket made up of ten anti-parallel

beta barrel sheets and to a helix-loop-helix cap.[114] Differences in function among this family arise with the tissue specific expression of each member.[114,155] FABP1, for example, is primarily expressed in the liver, but can also be found in the intestine, kidney, stomach, lung, and pancreas.[155–158] FABP4 is primarily expressed in adipocytes and macrophages while FABP5 is primarily expressed in epithelial tissues.[155,159] Other tissue specific FABPs include intestinal, muscle/heart, ileal, brain, peripheral nervous system, and testes.[154–156,160–162]

While all members of the FABP family bind and transport lipophilic molecules, there are broad physiological implications of protein activity due to their diverse tissue distribution. Adipose tissues express high levels of FABP4 and low levels of FABP5, indicating key roles for the proteins in triglyceride storage and inflammatory responses of adipose tissues.[159] Circulating FABP4 is significantly increased in breast cancer patients and FABP5 promotes lipid droplet formation and de novo fatty acid synthesis in breast and prostate cancer cell lines, suggesting that the fatty acid regulatory activities of these proteins may play a pro-oncogenic role.[163,164] Macrophages also expresses high levels of FABP4 along with FABP5, indicating additional roles for FABP4 and 5 in inflammatory cytokine production and endoplasmic reticulum (ER) stress response.[155,165–167] This means that both FABP4 and 5 play important roles in insulin resistance, diet-induced atherosclerosis, metabolic syndrome and inflammatory diseases.[115,158,166] FABP5 is also expressed at high levels in the brain where it serves to facilitate regulation of long chain fatty-acid supply.[155,168] FABP1, which is primarily expressed in the liver, varies slightly in its structure from many of the other FABPs and may therefore bind a wider variety of ligands.[155] This allows it to mediate long-chain fatty acid transport in beta-oxidation for

hepatic cells, giving it a crucial role in steatosis and nonalcoholic fatty liver disease. There is also evidence that FABP1 plays an important role in regulating endocannabinoid levels both endogenously and in response to phytocannabinoid treatment.[157,169,170]

In addition to their roles in fatty acid transport, FABP1, 4, and 5 are known to influence gene expression by association with peroxisome proliferator-associated receptors alpha, gamma, and delta, respectively.[125,171] Like, CRABP2, both FABP4 and FABP5 contain a non-classical tertiary NLS located on residues lysine 20, arginine 29, and lysine 30 or lysine 24, arginine 33, and lysine 34, respectively (Figure 1.4).[172,173] Because FABPs bind a wide variety of ligands, this tertiary NLS provides a structural control for the nuclear translocation of the proteins. Only ligands that fit into the binding pocket in such a way that allows proper formation of the NLS will initiate translocation. In the case of FABP5, those ligands that activate the protein are most often mono- or poly-unsaturated fatty acids that also serve as ligands for the nuclear receptor PPARδ.[173] The half-life of the rate of transfer of ligand between FABP5 and PPARδ is dependent on the donor/acceptor ratio, thereby indicating that FABP5-meidated activation of PPARδ requires direct protein-protein interaction. Furthermore, FABP5 must translocate to the nucleus to enact any sort of PPARδ transcriptional activity.[125] Together, these data provide strong reasoning for FABP5-mediation activation of PPARδ.

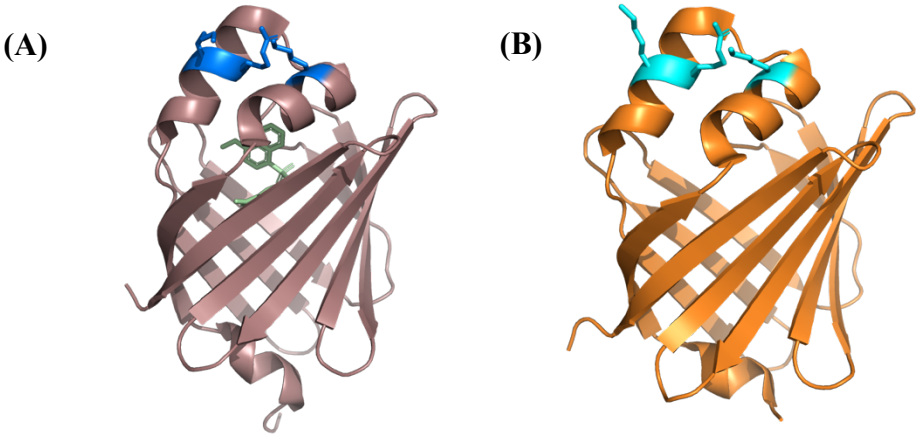

Figure 1.4 (A) Crystal structure of FABP4 in complex with 2-fluoro-5((4-methoxynapthalene)-1-sulfonamido) benzoic acid, shown in green, at 1.54 Å. The putative tertiary NLS, lysine 20, arginine 29, and lysine 30, is shown in dark blue. PDB ID: 5Y0F.[174] (B) Crystal structure of apo-FABP5 at 1.67 Å. Lysine 24, arginine 33, and lysine 34, shown in light blue, form the tertiary NLS. PDB ID: 4LKP.[173]

While the primary endogenous ligands of FABP5 are primarily saturated or unsaturated fatty acids, lipophilic atRA is also capable of binding to the protein with a K_D of about 34.8nM, an affinity well within the range of other FABP5 ligands.[89] FABP5 is known to enhance atRA-mediated activation of PPARδ, but does not induce any significant change in atRA-induced activation of RAR.[89] This indicates that FABP5 is capable of binding atRA, but is selective in specifically activating the PPARδ nuclear receptor. Genes targeted by PPARδ include those that promote lipogenesis, cell growth, proliferation and survival, indicating that activation of the receptor by holo-FABP5 can lead to tumor growth and development of atRA resistance.[175–181] Indeed, high expression of FABP5 is associated with poor survival in certain cervical, prostate, and breast cancers and treatment of cell lines and mouse models expressing a high level of FABP5 with atRA lead to increased tumor growth and poor survival via PPARδ activation.[89,182–184] Knockdown of FABP5 in the MMTV-Neu breast cancer mouse model significantly

delayed tumor growth and increase mice survival. In addition, expression levels of PPARδ target genes and of members of the HER2 signaling pathway in tumors from FABP5 *null* MMTV-Neu mice were significantly .[185] The accumulated data strongly suggests that the binding proteins CRABP2 and FABP5 play a key role in channeling atRA between RAR and PPARδ and therefore regulate the function of atRA in the cell.

1.4 The Dual Activity of atRA in Cancer Cells

RAR and PPARδ regulate different sets of genes and therefore atRA exerts distinct, and sometimes opposing, activities in cells where it activates RAR versus cells where it signals through PPARδ. While activation of RAR suppresses cell proliferation, PPARδ targets genes that support cell growth and survival. The partitioning of atRA between its receptors is controlled by CRABP2 and FABP5 and consequently, RA activates RAR in cells that express CRABP2 but signals through PPARδ in cells that highly overexpress FABP5 (Figure 1.5).

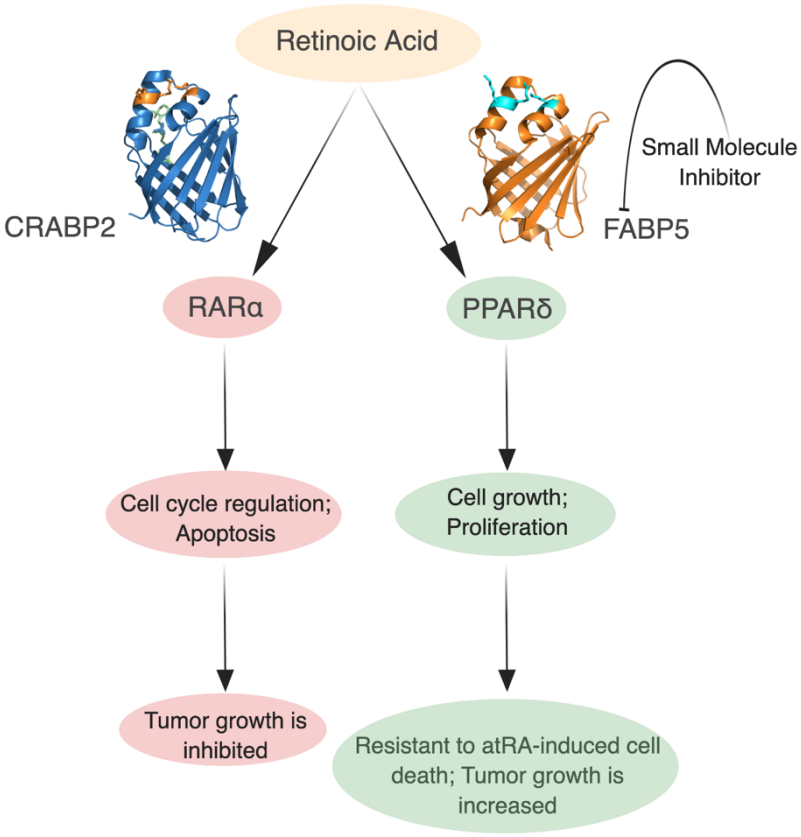

Figure 1.5 The dual signaling pathways of atRA. When there is a high expression level of CRABP2, the iLBP will deliver atRA to the RAR/RXR heterodimer where it will initiate the transcription of genes associated with cell cycle regulation and apoptosis. In the context of cancer treatment, this gives the net effect of tumor inhibition. If the expression level of FABP5 is much higher than that of CRABP2, however, atRA will be delivered to PPARδ where it will initiate the transcription of genes that promote cell growth and proliferation. This renders a net effect of tumor growth. The central hypothesis of this project is that if a small-molecule inhibitor is targeted to FABP5 in cases of high expression, atRA will fail to activate PPARδ via FABP5 and may even re-direct its transcriptional activity to the CRABP2/RAR signaling pathway.

atRA signals through the FABP5/PPARδ pathway and displays pro-oncogenic activities only in cells with low CRABP2/FABP5. For example, tumors of the MMTV-*Neu* mouse model of breast cancer express a high level of FABP5 and low levels of CRABP2 and display a growth response when treated with atRA. Yet, the surrounding

normal mammary tissue expresses a higher level of CRABP2 relative to the expression of FABP5.[89] When CRABP2 is overexpressed in these tumors *in vivo*, growth is delayed and overall survival of the mice is improved.[147]

Both NaF cells, mouse mammary carcinoma cells derived from MMTV-*Neu* tumors, and HaCaT cells, a human keratinocyte cell line, express a high level of FABP5 while expressing little to no CRABP2. When either of these cell lines are treated with the apoptosis inducer tumor necrosis factor α (TNFα), apoptosis is induced as expected. When the cells are treated with atRA and TNFα concurrently, the atRA is able to protect the cells from the proapoptotic effects of TNFα. Furthermore, when CRABP2 is overexpressed or FABP5 expression is eliminated in these cells, the protective effects of atRA against TNFα are eliminated.[89] This effect is reversed in the human mammary carcinoma cell line MCF7, which expresses a high level of CRABP2 relative to FABP5. Treatment of MCF7 cells with the apoptosis-inducing agent tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in the presence or absence of atRA. Yet, when CRABP2/FABP5 ratio in the cells is altered by silencing CRABP2 and overexpressing FABP5, TRAIL treatment no longer induces apoptosis in the presence of atRA. This is due to the pro-proliferative action of atRA when FABP5 is highly overexpressed compared to CRABP2.[89] The differential expression levels of CRABP2 and FABP5 correspond to a consequential rise or fall in the activation of RAR or PPARδ target genes in response to atRA treatment.[86,89] Taken together, available information indicates that FABP5 functions as an oncogene and that it facilitates tumor growth by diverting atRA from RAR to PPARδ. Inhibition of FABP5 is thus expected to

re-direct atRA from PPARδ to RAR, thereby sensitizing carcinoma cells to atRA-induced growth inhibition.

1.5 Targeting FABP5 to Prevent atRA-Mediated Activation of PPARδ

The central hypothesis of this project proposes to test the efficacy of using a small-molecule inhibitor to target FABP5 in cancers that express high levels of the protein and are resistant to atRA for inhibition of cancer cell growth. Inhibition of FABP5 is expected to have the dual activity of inhibiting the transcriptional activity of PPARδ (Figure 1.5) and re-directing atRA toward CRABP2/RAR and activating this anti-carcinogenic signaling pathway. In order for any small molecule to be considered as a potential FABP5 inhibitor it must meet two criteria: 1) the molecule cannot independently activate PPARδ and 2) it must effectively bind FABP5 in such a way that prevents FABP5-mediated activation of the PPARδ signaling pathway.

A logical first place to look for potential FABP5 inhibitors is among its natural ligands: long-chain fatty acids.[186] While both saturated and unsaturated long-chain fatty acids (LCFA) bind to purified human FABP5 with similar affinity, unsaturated LCFA, linoleate in particular, dramatically increase PPARδ activation when in the presence of atRA. This effect is eliminated when FABP5 expression is knocked down.[187] This regulation of FABP5 activation can be partially explained by the U-shape of unsaturated LCFA facilitating proper formation of the tertiary NLS located on the protein's alpha helical cap.[173] Saturated LCFA, on the other hand, hinder carcinoma cell growth via FABP5 inhibition.[187] When carcinoma cell lines that express a high level of FABP5, such as MDA-MB-231 and PC3M, are treated with palmitate, a saturated LCFA, there is a

significant decrease in cell proliferation. This effect disappears when FABP5 expression is eliminated. Palmitate also reduces the expression of PPARδ target genes while increasing the activation of RAR target genes in NaF cells. Additionally, palmitate reduces growth and shifts atRA signaling from PPARδ to RAR in tumors from mice fed palmitate-supplemented diets.[187] Taken together, these data suggest that palmitate may be an excellent candidate FABP5 inhibitor given its ability to effectively bind FABP5 and divert atRA signaling from PPARδ to RAR without independently activating the PPARδ signaling pathway.

While palmitate shows promise as an FABP5 inhibitor, several factors complicate its use in a wider pharmacological context. There is some evidence that limited low-doses of palmitate may improve insulin sensitivity and inflammatory response in endothelial cells, but the potential health risks of palmitate far outweigh the potential benefits.[188] Palmitate is metabolized rapidly in the body and any pharmacologically relevant dose of the fatty acid is therefore quite high.[189–193] Excess circulation of fatty acids, and saturated fatty acids in particular, is associated with non-alcoholic fatty liver disease, insulin resistance, and diabetic nephropathy.[194–196] Furthermore, saturated fatty acids like palmitate have been shown to have a more serious negative effect on overall health, including an increase in apoptosis in hepatic cells and increased production of reactive oxygen species when compared to treatment with unsaturated fatty acids.[197–199] Given the lipotoxicity associated with high doses of palmitate, it stands to reason that an alternative small-molecule inhibitor for FABP5 should be explored. The goal of this project is to screen potential inhibitors for a compound that will effectively bind FABP5 and prevent activation of PPARδ in those cancers that express a high level of FABP5 relative to

CRABP2. These compounds must meet all criteria for an effective FABP5 inhibitor. While the pharmacology of these potential inhibitors falls outside of the scope of this project, these new proposed inhibitors will ideally avoid the wide-spread negative systemic effects that are present with palmitate treatment.

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CHAPTER 2. FABP5 INHIBITOR SCREENING

2.1 INTRODUCTION

All-trans retinoic acid (atRA) is the major active metabolite of vitamin A and is responsible for enacting a wide variety of biological effects. Canonically, atRA will bind the nuclear receptor retinoic acid receptor (RAR) which forms a heterodimer with the retinoid X receptor (RXR) and binds directly to a specific DNA sequences called retinoic acid response elements (RAREs). Once liganded, the activated RAR/RXR heterodimer will go on to initiate transcription of RAR target genes, including those associated with cell cycle arrest and apoptosis. This anti-proliferative activity of atRA makes the compound a promising treatment for some cancers such as acute promyelocytic leukemia for which atRA is a first-line treatment. However, despite the therapeutic potential of atRA in some cancer types, other cancers can exhibit resistance and express increased growth in response to atRA treatment. This seemingly contradictory effects of atRA was found to be as a result of the ability of atRA to activate an additional nuclear receptor, peroxisome proliferator activated receptor δ (PPARδ). Like RAR, PPARδ forms a heterodimer with RXR and binds directly to specific PPAR response elements (PPRE).[1] PPARs have a ligand binding site that is 3-4 times larger than those of other nuclear receptors, thus allowing for a wide variety of natural ligands, including long chain unsaturated fatty acids and atRA.[2,3] Unlike other isoforms of PPAR, PPARδ is widely expressed in all tissues.[2] Once liganded, PPARδ activates transcription of target genes that are completely different from those of RAR and accordingly, responsible for different cellular functions. Some of PPARδ target genes include those involved in lipid uptake, metabolism, proliferation and survival, thus promote carcinogenic activities.[2,4]

Although Both RAR and PPARδ binds, the K_D for atRA binding to PPARδ is about 15nM-approximately 10 orders of magnitude lower than the K_D for RA binding to RAR.[3] This means that there must be a factor outside of receptor-ligand binding affinity contributing to the dual effects of atRA.

The partitioning of atRA between RAR and PPARδ was found to be regulated by two intracellular lipid-binding proteins (iLBPs), cellular retinoic acid binding protein 2 (CRABP2) and fatty acid binding protein 5 (FABP5). In canonical atRA signaling, CRABP2 binds atRA in the cytoplasm and delivers it to the nucleus.[5] However, another iLBP, FABP5, is capable of binding lipid-like atRA. FABP5 is a small 15kDa protein with a beta-barrel fold binding site and an alpha-helical cap.[6] The alpha-helical cap contains a nuclear localization signal (NLS) comprised of two solvent-exposed lysines and an arginine that, once activated, will direct FABP5 to the nucleus.[6] When L-shaped saturated long chain fatty acids bind FABP5, this NLS is not properly formed and FABP5 translocation is prevented. The U-shape of poly-unsaturated fatty acids, however, fits snugly inside of the FABP5 and allows proper NLS formation.[6] This allows for ligand-specific regulation of FABP5. Once FABP5 binds and activated by atRA, it delivers the ligand specifically to PPARδ.[4] Accordingly, cancer cells exhibiting high expression level of FABP5 relative to CRABP2 are resistant to atRA and will respond to atRA treatment with increased proliferation.[4] In breast and ovarian cancers, high expression levels of FABP5 are associated with increased tumor growth and metastasis and significantly reduced long-term survival.[7,8] Furthermore, high FABP5 levels promote growth and metastasis in colorectal, cervical, and prostate cancers.[9–11] The central hypothesis of this work centers on the ability to prevent the activation of FABP5 by using a small molecule

inhibitor. An inhibitor targeted to FABP5 should prevent atRA from activating PPARδ and its target pro-proliferative genes and could re-direct atRA to bind CRABP2, thereby activating the RAR signaling pathway. The essential criteria for a suitable FABP5 inhibitor are: 1) any candidate compound must bind FABP5 with high affinity and 2) the inhibitor cannot independently activate PPARδ. Previous studies have shown that palmitate, a 16-carbon saturated fatty acid, binds FABP5 with high affinity and is able to inhibit cell proliferation both *in vitro* and *in vivo*.[12] However, given the rapid metabolic consumption of palmitate, any pharmacologically relevant does of the compound would be toxic.[13,14] It is therefore imperative to explore new potential inhibitors for FABP5. A natural starting point when investigating potential protein inhibitors is to look to the natural ligands of that target. FABPs primarily bind and chaperone insoluble long-chain free fatty acids in the cytoplasm, so we first looked for compounds that closely resemble long-chain fatty acids.[15,16] There are also several reported synthetic FABP4/FABP5 and FABP7/FABP5 dual inhibitors that contain multiple rings, so this provides another structural motif from which to choose potential inhibitors.[15,17–20]

Among the library of candidate inhibitors, compounds **1**, **2**, and **3** are naturally occurring long-chain fatty acids. Compound **1** is a 16-carbon furanyl fatty acid found most commonly in the lipid contents of fish, but can also be found in microorganisms, algae and other plants.[21] Compound **2**, or eladic acid, is the trans isomer of oleic acid and is found naturally in caprine and bovine milk and some meats.[22,23] It has demonstrated some anti-tumorigenic and pro-inflammatory activity in addition to some anti-proliferative activity in hepatocytes.[24,25] Compound **3**, punicic acid, is derived from pomegranate and has some anti-metastatic properties in hepatocellular carcinoma cell

lines.[15,16] Compounds **4**, **5**, **7**, and **8** are not naturally occurring, but are known to bind other FABPs or mimic those compounds that do.[26–28] The remaining compounds, **6**, **9**, **10** and **11**, are hits from a high-throughput screening of potential FABP5 ligands commissioned from the University of Cincinnati Drug Discovery Center.

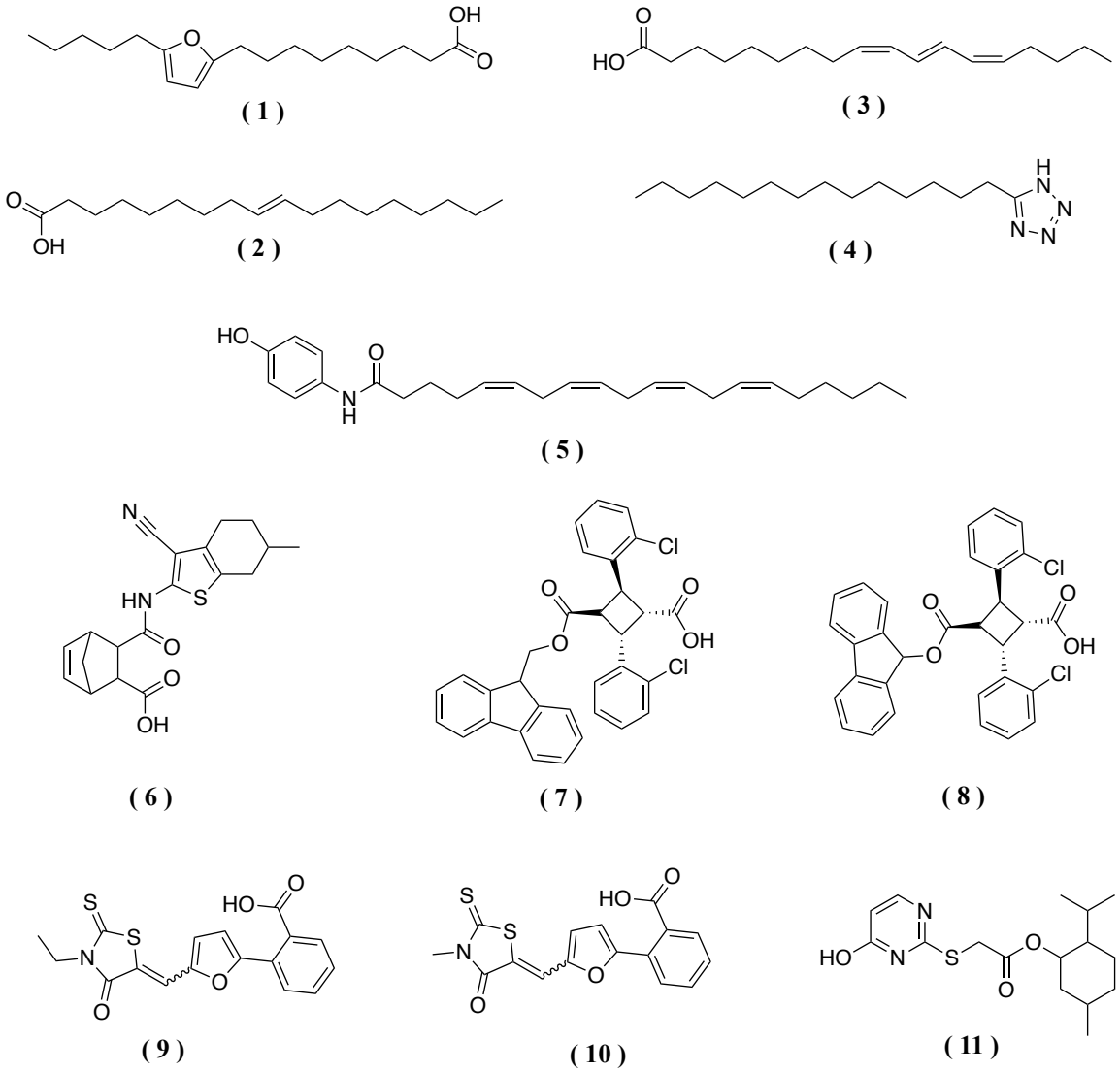


Figure 2.1 Library of candidate FABP5 inhibitors. The potential inhibitors can be roughly divided into fatty-acid like molecules (compound 1-5) and multi-ring compounds (compounds 6-11).

2.2 Results and Discussion

The first criteria for a good FABP5 inhibitor is that the candidate compound does not independently activate PPARδ. If this independent activation occurs, any subsequent FABP5 binding activity will be meaningless. Thus, the first step in screening candidate FABP5 inhibitors is to check for PPARδ activation via PPRE transactivation assay. If the inhibitors are able to bind to FABP5 with a reasonably low K_D and do not activate PPARδ, the compound must be applied to a cancer cell line in order to evaluate how a compound bound to FABP5 will impact PPARδ signaling, and therefore proliferation, of the cells. For this study, we chose MDA-MB-231 cells, a human triple-negative breast cancer cell line, to evaluate our candidate inhibitors. This cell line expresses a high level of FABP5, so it is a good model for manipulating the FABP5/PPARδ signaling balance. Triple-negative breast cancer is particularly aggressive and difficult to treat, so an inhibitor targeting FABP5 to reduce malignant properties of the cancer could provide a useful potential therapeutic strategy.[29,30] In addition to 231 cells, we chose MCF7 cells, a human breast cancer cell line that expresses a low level of FABP5 and normal human mammary cells, HMEC, to demonstrate the FABP5-specific activity of these inhibitors.

2.2.1 PPRE Transactivation Assays

Because all of the potential inhibitors are reported or expected to bind FABP5 with reasonable affinity, the first step in evaluating their potential is to ensure that they do not independently activate PPARδ. If the compounds are able to activate PPARδ, either via FABP5 or another route, any anti-proliferative effects due to FABP5 inhibition will be rendered moot. When PPARδ activation is evaluated via a PPRE luciferase

transactivation reporter assay, 20μM treatment with compounds **3**, **9**, **10** or **11** induces notable activation of PPARδ, eliminating these compounds from consideration as FABP5 inhibitors (Figure 2.2). While compound **3**, punicic acid, is known to reduce pro-proliferative and metastatic properties in cancer cell lines, its poly-unsaturated structure likely allows it to act as a pan-PPAR agonist and therefore must initiate anti-proliferative signaling outside of the FABP5 pathway.[15–17,31,32] Additionally, compounds **9** and **10** are quite similar to other rhodanines that function as known PPARγ agonists, so their subsequent activation of PPARδ is unsurprising.[33]

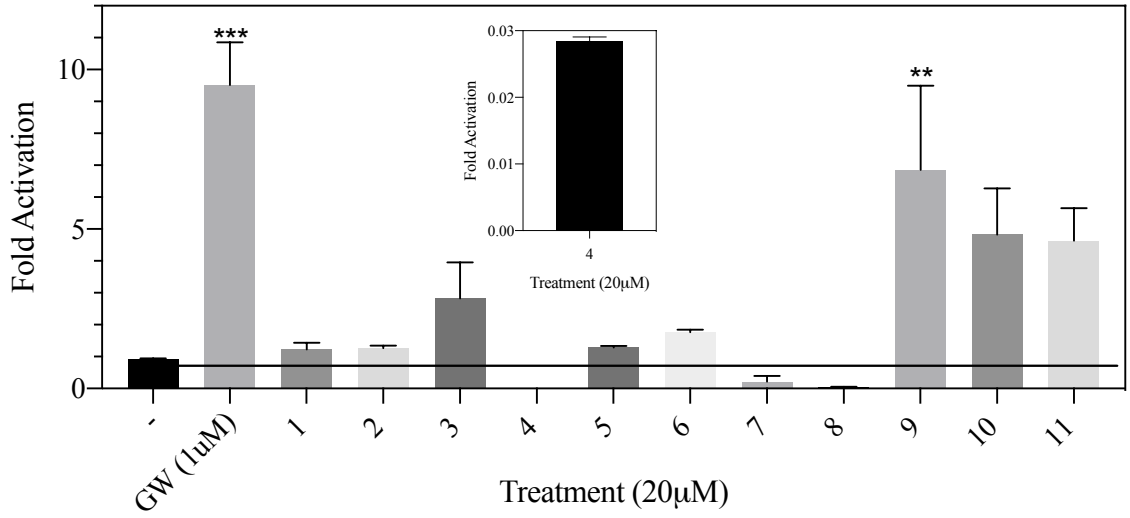


Figure 2.2 PPRE Transactivation assays of potential FABP5 inhibitors. Cos7 cells were transfected with an expression vector for PPARδ along with a PPRE-Luciferase reporter vector and an expression vector for β-galactosidase as transfection control. Transfected cells were treated with 1μM PPARδ agonist GW501516 or 20μM FABP5 inhibitor candidate for 18hrs. Luciferase was calculated relative to β-galactosidase activity. n=2, *$p<0.05$, **$p<0.005$

2.2.2 Proliferation Assays

When FABP5 is inhibited, PPARδ activation, and therefore cell proliferation, is truncated. When the remaining candidate inhibitors are incubated with FABP5-rich 231 cells for four days, compounds **1**, **2**, **4**, **7** and **8** do not result in any significant decrease in proliferation (Figure 2.3). While these compounds do not independently activate PPARδ, they are not able to sufficiently manipulate the FABP5/PPARδ signaling balance in such a way that reduces proliferation and are therefore disqualified as FABP5 inhibitors. Treatment with compound **5** decreases the proliferation of 231 cells in a dose-responsive manner to a minimum of 21.8% proliferation at 20μM treatment. Compound **6** is able to significantly reduce proliferation to about 51% with 20μM treatment, making both compounds promising candidate inhibitors (Figure 2.3).

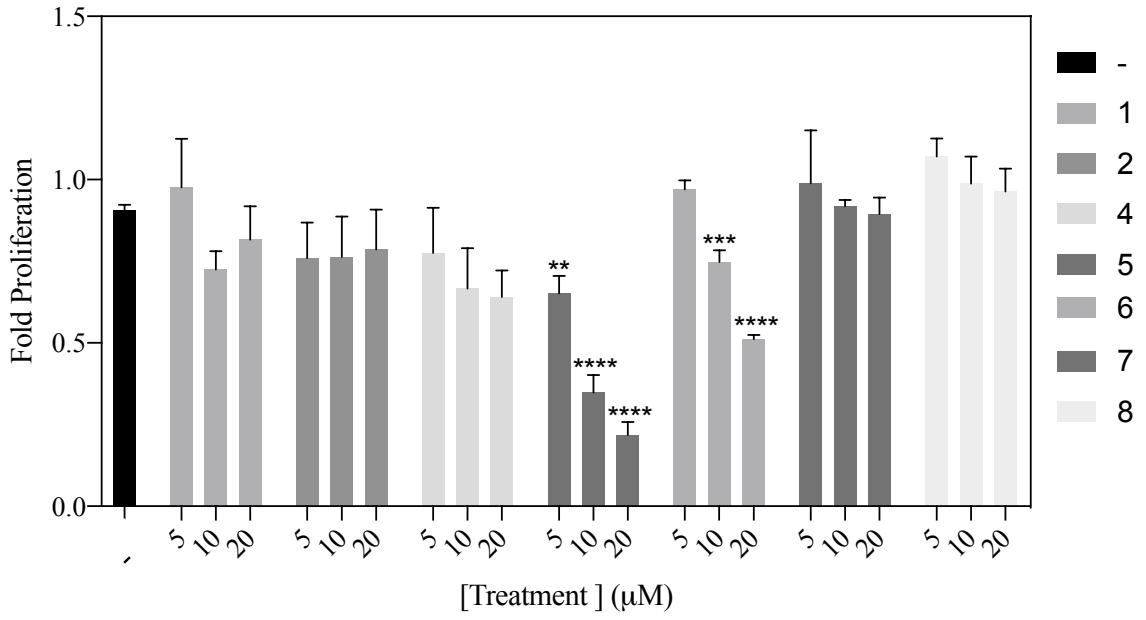


Figure 2.3. Proliferation in MDA-MB-231 cells treated with candidate FABP5 inhibitor compounds. Cells were incubated with 5, 10 or 20μM compound for 4 days and then cell viability was evaluated with PrestoBlue Cell Viability reagent. n=3, **p<0.005, ***p<0.0005,****p<0.0001.

2.2.3 Choosing a Lead Compound

Among the compounds tested from the initial screening library, only compounds **5** and **6** are able to effectively bind purified FABP5 and reduce cell proliferation in FABP5-rich 231 cells while not independently activating PPARδ. In order to further evaluate how these compounds manipulate the CRABP2/FABP5 signaling ratio, 231 lines stably expressing FABP5 shRNA or a control shRNA were generated. Cells were incubated with compounds **5** and **6** for 6 hours. Following incubation, RNA was extracted and the expression of PPARδ and RAR target genes were evaluated via qRT-PCR. Treatment of the control 231 line with 5, 10, or 20 μM compound **5**, resulted in a dose responsive decrease in the expression of PPARδ target genes *Pdpk1* and *Plin2* and a simultaneous dose-responsive increase in the expression of RAR target genes *Rarβ* and *Cyp26a1*(Figure 2.4a and b). A similar dose-responsive increase in PPARδ target genes and a decrease in RAR target genes (Figure 2.4 c and d) is observed in the 231control line treated with compound **6**. To test that the effect of the compounds on PPARδ and RAR targets is FABP5-dependent 231 stable line in which FABP5 was knockdown was utilized. The effect of compound 5 on PPARδ and RAR targets was markedly attenuated, Yet, no difference was observed in levels of PPARδ targets in the presence or absence of FABP5 in response to compound 6 treatment. Together, these data indicate that both compounds **5** and **6** are able to effectively prevent activation of PPARδ and induce activation of RAR in a dose-responsive manner, but the role of FABP5 remains unclear in regards to compound **6**. Compound **5** gene expression data is provided courtesy of Dr. Liraz Levi.

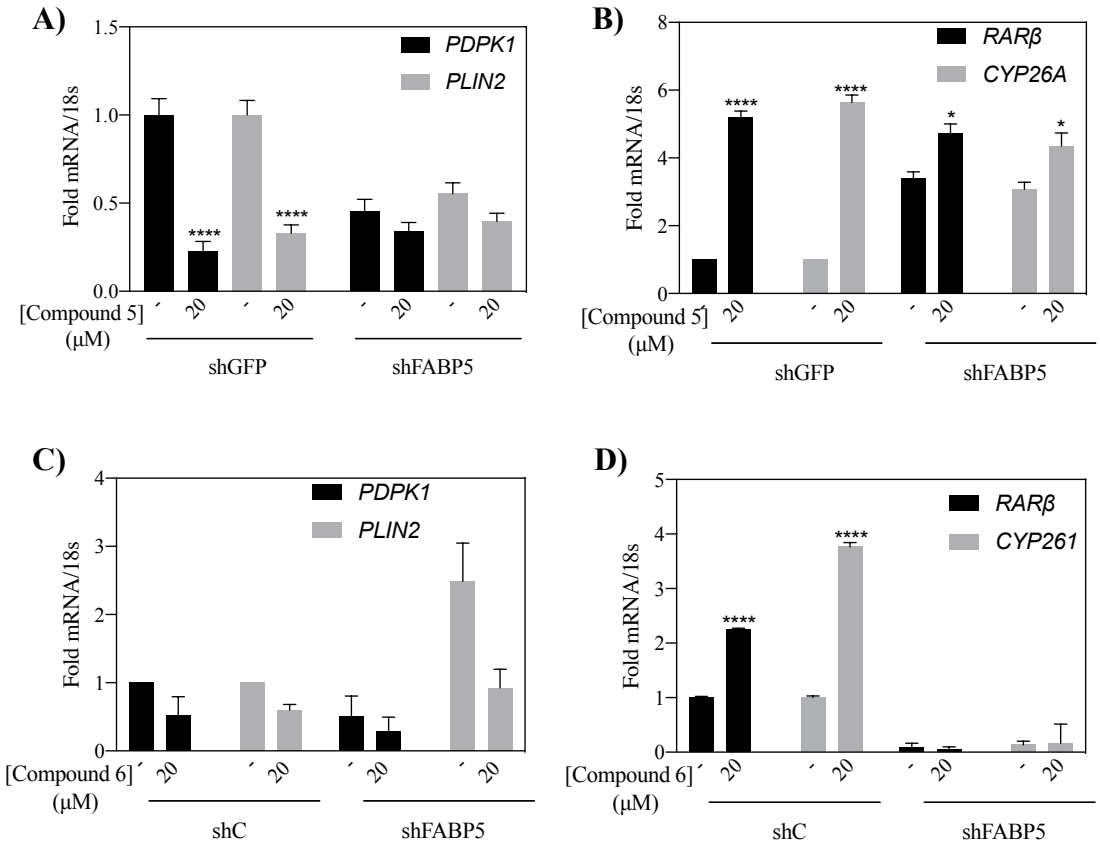


Figure 2.4 Expression of PPARδ and RAR target genes in 231 cells transfected with a control vector or a vector to knockdown FABP5 expression and incubated with compounds **5** and **6**. In those cells incubated with compound **5**, PPARδ target genes decrease only in control cells (A) with no change when FABP5 expression is eliminated. A similar increase in RAR target genes is also observed for compound **5** (B). While a similar pattern is seen for RAR targets genes after incubation with compound **6** (D), PPARδ target genes still decrease after FABP5 is knocked down (C). Compound **5** gene expression data courtesy of Dr. Liraz Levi. n=3, *p<0.05, ****p<0.0001

In addition to their ability to manipulate PPARδ and RAR signaling via FABP5, both compounds **5** and **6** demonstrate specificity for cells that express a high level of FABP5. The breast cancer cell line MCF7 and normal human mammary cells (HMEC), both of which express low FABP5, do not respond to incubation with either compound **5** or compound **6** (Figure 2.5a and 2.5b). This demonstrates that the anti-proliferative effect seen from these compounds depends upon the presence of FABP5-thus validating our

hypothesis that inhibiting FABP5 with a small molecule inhibitor will inhibit proliferation of the tumor cells via reduction in PPARδ signaling. Proliferation data in MCF7 and HMEC cell lines are courtesy of Dr. Liraz Levi.

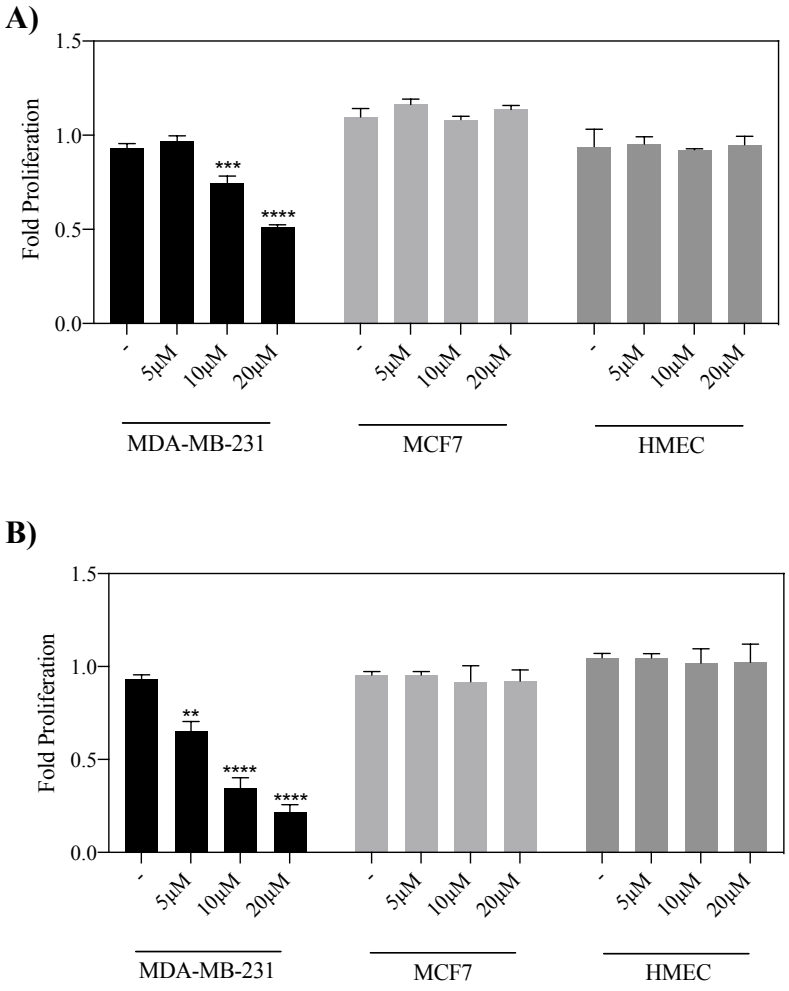


Figure 2.5. Proliferation assays of compounds 5 (A) and 6 (B) with MDA-MB-231, MCF7, and HMEC cell lines. Both compounds only induce a reduction in proliferation in the FABP5-rich MDA-MB-231 cells. There is no effect on proliferation in the cell lines that express low FABP5. Proliferation data in MCF7 and HMEC lines are courtesy of Dr. Liraz Levi. n=3, **p<0.01, ***p<0.001, ****p<0.0001

2.3 Conclusions

While compound **5**, also known as AM404 or N-arachidonylaminophenol, is certainly a viable candidate for FABP5 inhibition and does exhibit promising anti-cancer properties, it is an active metabolite of acetaminophen and is thought to be responsible for some or all of its analgesic effects.[26,27] It is a known modulator of anandamide transport and the endocannabinoid system and has other broad neurological effects, including the elimination of fear memory responses and signs of anxiety in conditioned rats.[18–20,34,35] This broad bioactivity will likely complicate any possible clinical application of this potential FABP5 inhibitor, so while pursuit of this candidate is certainly worthwhile, its larger bioactive potential disqualifies it as an FABP5 inhibitor for the purposes of this study. That leaves compound **6**, a commercial compound, as the most promising candidate FABP5 inhibitor from the original screening library.

2.4 Experimental Methods

2.4.1 General Experimental Methods

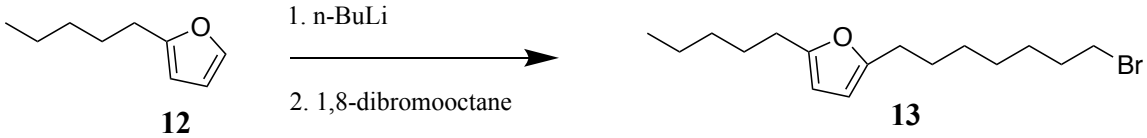
MDA-MB-231, MCF7, and COS7 cells were cultured in DMEM with L-glutamine and 4.5g/L glucose without sodium pyruvate supplemented with 10%FBS and 100IU penicillin/100mg/mL streptomycin. HMEC cells were cultured in HMEC ready medium. All cells were cultured in a humidified atmosphere at 37^{o}C and 5% CO_2. Absorbance, fluorescence, and luminescence readings were performed on a Molecular Devices SpectraMax i3x Multimode detection platform. All statistical analyses were calculated with GraphPad Prism software.

2.4.2 Reagents and Chemicals

MDA-MB-231, MCF7, Cos7, and HMEC cells were purchased from ATCC. DMEM, penicillin-streptomycin 100x solution, and 2.5% trypsin solution were purchased from Corning. US-origin FBS was purchased from VWR. PrestoBlue Cell Viability reagent, TriZol, and B-PER were purchased from ThermoFisher Scientific. IPTG, ampicillin, and imidazole were purchased from Sigma Aldrich. Ni Sepharose 6 FastFlow Beads were purchased from GE Life Science. The luciferase reporter assay system was purchased from Promega. iScript cDNA synthesis kit was purchased from BioRad. Compound **2,** elaidic acid, was purchased from Millapore Sigma. Compound **3,** punicic acid, was purchased from Larodan. Compound **5,** AM404, was purchased from Fisher Scientific and compound **6** was purchased from The Enamine Store. All reagents used for synthesis were purchased and used without further purification.

2.4.3 Synthesis of 8-(5-Pentylfuran-2-yl)Octanoic Acid (1):

- **Synthesis of 2-(7-Bromoheptyl)-5-Pentylfuran (13)**



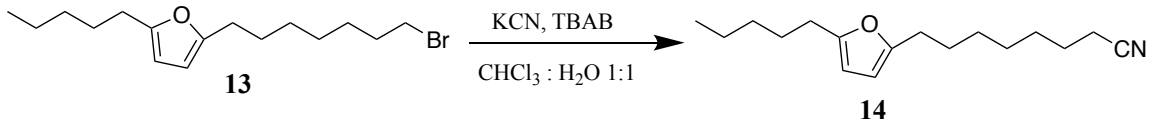
12 **13**

2-pentylfuran **(12)** (2.17g, 15.7mmol) was added to 30mL anhydrous THF and flushed with nitrogen. A 2.5M solution of n-BuLi in hexanes (4.71g, 17.3mmol) was added dropwise at 0^{o}C. The solution was allowed to warm to room temperature and stirred for 2 hours. 1,8-dibromooctane (13.0g, 47.8mmol) was added dropwise to the solution and allowed to stir at 0^{o}C overnight. The reaction mixture was quenched with an excess of 1-

butanol and poured over ice. The product was extracted with ether, washed with brine, and dried over sodium sulfate. Excess solvent was removed under vacuum and the resulting crude 2-(7-bromoheptyl)-5-pentylfuran **(13)** was purified using fractional distillation at 0.1 Torr for a yield of 62.6%.

^{1}H NMR (500Mz, $CDCl_3$): δ 5.85 (s, 2H), 3.50 (t, J=7.5Hz, 2H), 2.58 (t, *J*=7.7Hz, 4H), 1.82 (t, *J*=7.0Hz, 2H), 1.70-1.58 (m, 4H), 1.39-1.30 (m, 10H), 0.92 (t, *J*=1.4Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 150.3, 104.9, 104.8, 34.1, 33.7, 32.6, 29.05, 29.02, 28.69, 28.62, 28.08, 28.04, 28.01, 27.85, 22.4, 17.0, 14.0.

- **Synthesis of 8-(5-pentylfuran-2-yl)octanenitrile (14)**

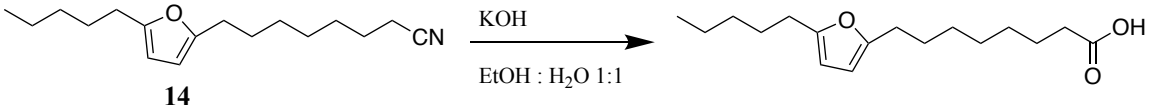


13 **14**

2-(7-bromoheptyl)-5-pentylfuran **(13)** (2.00g, 6.07mmol), potassium cyanide (3.99g, 60.7mmol), and tetra-n-butylammonium bromide (391mg, 1.214mmol) were combined in 12mL $CHCl_3$ and 12mL H_2O. The reaction mixture was heated to reflux overnight. The crude product was extracted three times with DCM. The organic layers were combined, washed with brine, dried over sodium sulfate, and dried under vacuum. 8-(5-pentylfuran-2-yl)octanenitrile **(14)** was purified by via silica column chromatography in 5:95 ethyl acetate:hexanes for a yield of 44.81%.

^{1}H NMR (500Mz, $CDCl_3$): δ 5.85 (s, 2H), 2.58 (t, *J*=7.7Hz, 4H), 2.31 (t, *J*=7.1Hz, 2H), 1.70-1.58 (m, 6H), 1.49-1.42 (m, 2H), 1.39-1.30 (m, 12H), 0.92 (t, *J*=1.3Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 154.5, 154.2, 119.7, 104.9, 104.8, 31.4, 29.05, 29.02, 28.69, 28.62, 28.08, 28.04, 28.01, 27.85, 25.3, 22.4, 17.0, 14.0.

- **SYNTHESIS OF 8-(5-PENTYLFURAN-2-YL)OCTANOIC ACID (1)**



14 **1**

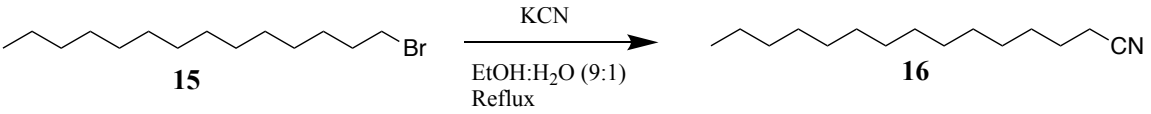
8-(5-pentylfuran-2-yl)octanenitrile **(14)** (740mg, 2.69mmol), potassium hydroxide (1.48g, 26.9mmol), 6mL ethanol and 6mL of water were combined in a round bottom flask and heated to reflux overnight. The crude product mixture was acidified to pH 4 with 1M HCl and extracted three times with ether. The organic layer was washed with brine, dried over sodium sulfate, and dried under vacuum. 8-(5-pentylfuran-2-yl)octanoic acid **(1)** was purified via silica column in 25:75 ethyl acetate:hexanes with a 66.9% reaction yield, % overall yield.

^{1}H NMR (500Mz, $CDCl_3$): δ 11.7 (s, 1H), 5.75 (s, 2H), 2.47 (t, *J*=7.6Hz, 4H), 2.26 (t, *J*=7.5Hz, 2H), 1.58-1.47 (m, 6H), 1.29-1.20 (m, 12H), 0.82 (t, *J*=7.1Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 179.5, 153.5, 153.4, 103.8, 103.7, 33.09, 30.4, 28.13, 28.10, 28.0, 27.08, 27.02, 26.8, 23.6, 21.4, 12.9.

All synthetic protocols and characterizations for compounds 4, 7, 8, 9, 10 and 11 were designed and performed by Yuzhi Shang and Yongchun Hou.

2.4.4 SYNTHESIS OF 5-TETRADECYL-1*H*-TETRAZOLE (4):

- **Synthesis of Pentadecanenitrile (16)**



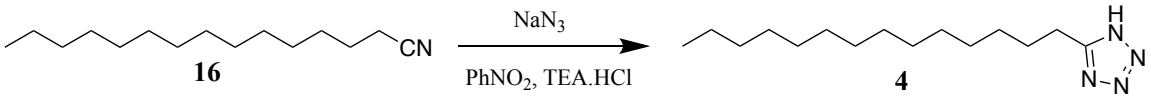
15 **16**

1-bromotetradecane (**15)** (2.0mL, 6.72 mmol) and KCN (1.31g, 20.2 mmol) were added to EtOH (18mL) and water (2mL) in 100mL round bottom flask at 25^{0}C with stirring. The reaction mixture was heated to 32^{0}C and allowed to reflux for 22 hrs. The reaction was quenched with water and extracted twice with ethyl acetate. The organic layers were

washed and dried with sodium sulfate, filtered, and dried under vacuum. 1.365 g of pentadecanenitrile **(16)** was obtained at 91% yield.

^{1}H NMR (500MHz, $CDCl_3$): δ 2.33 (t, *J*=7.1Hz, 2H), 1.65 (m, 2H), 1.26 (m, 22H), 0.88 (t, *J*=7.1, 8.0Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 119.2, 17.3, 31.9, 29.6, 29.3, 28.8, 28.5, 25.5, 22.7, 14.1.

- **Synthesis of 5-tetradecyl-1*H*-tetrazole (4)**

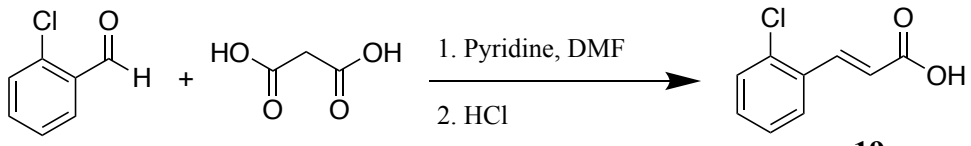


Pentadeanenitrile **(16)** (700mg, 3.13mmol), sodium azide (269 mg, 4.15 mmol), and trimethylamine HCl (571mg, 4.15mmol) were added to 12mL nitrobenzene in a 100mL round bottom flask. The mixture was sonicated and then refluxed at 24^{0}C for 18 hrs. The reaction mixture extracted 3 times with water. Aqueous layers were combined and washed twice with ether. The aqueous layers were combined and acidified with HCl to pH=2. The precipitate was filtered and washed twice with water. 5-tetradecyl-1*H*-tetrazole **(4)** was obtained at 6.5% yield (61.7mg). Overall reaction yield 5.9%.

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.83 (t, *J*=7.1Hz, 2H), 1.66 (t, *J*=7.1, 2H), 1.28 (m, 2H), 1.26 (m, 20H), 0.85 (t, *J*=8.0Hz, 3H); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 159.2, 31.9, 29.6, 29.3, 23.1, 22.7, 14.1.

2.4.5 SYNTHESIS OF (1*S*, 2*S*, 3*S*, 4*S*)-2,4-BIS(2-CHLOROPHENYL)-3-(((2,9-DIHYDRO-1H-FLUOREN-9-YL)METHOXY)CARBONYL)CYCLOBUTANE-1-CARBOXYLIC ACID (7):

- **Synthesis of (E)-3-(2-chlorophenyl) acrylic acid (19)**

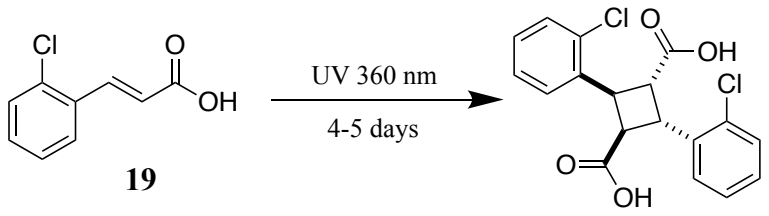


17 **18** **19**

2-chlorobenzaldehyde **(17)** (2g, 14.23 mmol), malonic acid **(18)** (4.44g, 42.69 mmol), and dry pyridine (1.15mL) were added 15mL DMF. The reaction mixture was refluxed at 90^0C overnight. The reaction was quenched with 30mL water. The mixture was placed in an ice bath and HCl was added until pH=1. The product was washed 3 times with water until pH=3-4 and dried under vacuum. (E)-3-2(Chlorophenyl) acrylic acid **(19)** was obtained at 87.9% yield.

^{1}H (500 MHz, $CDCl_3$): δ 8.23 (d, *J*=15.1 Hz, 1H), 7.67 (dod, *J*=7.5, 1.5 Hz, 1H), 7.45 (dod, *J*=7.5, 1.5 Hz, 1H), 7.34 (m, 2H), 6.44 (d, *J*=15.1Hz, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 171.5, 147.7, 134.0, 133.0, 129.9, 129.1, 127.9, 126.6, 116.5.

- **Synthesis of (1*R*, 2*R*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)cyclobutane-1,2-dicarboxylic acid (20)**

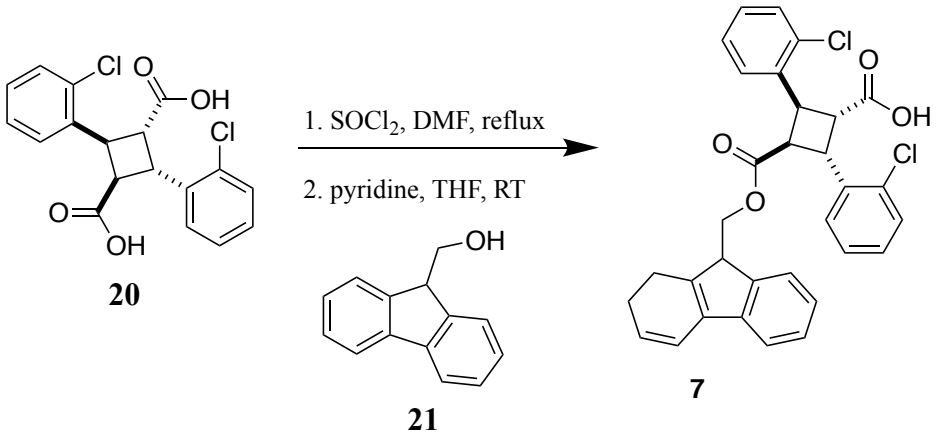


19 **20**

(E)-3-2(Chlorophenyl) acrylic acid **(19)** (500mg, 1.37mmol) was added to a quartz round bottom flask with 20mL hexanes. The mixture was sonicated for 20 seconds and mixed well. The reaction was exposed to a UV lamp at 360nm for 4 days. The crude product was purified by CombiFlash column using DCM:MeOH 100:1-100:4.5 with ten drops of formic acid/100mL. (1*R*, 2*R*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)cyclobutane-1,2-dicarboxylic acid **(20)** was obtained at 90.7% yield.

^{1}H (500 MHz, $CDCl_3$): δ 7.21 (m, 2H), 7.05 (m, 6H), 4.98 (d, *J*=7.0Hz, 2H), 3.90 (d, *J*=7.0Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 172.6, 136.8, 133.4, 129.5, 128.5, 127.3, 126.6, 44.7, 38.3.

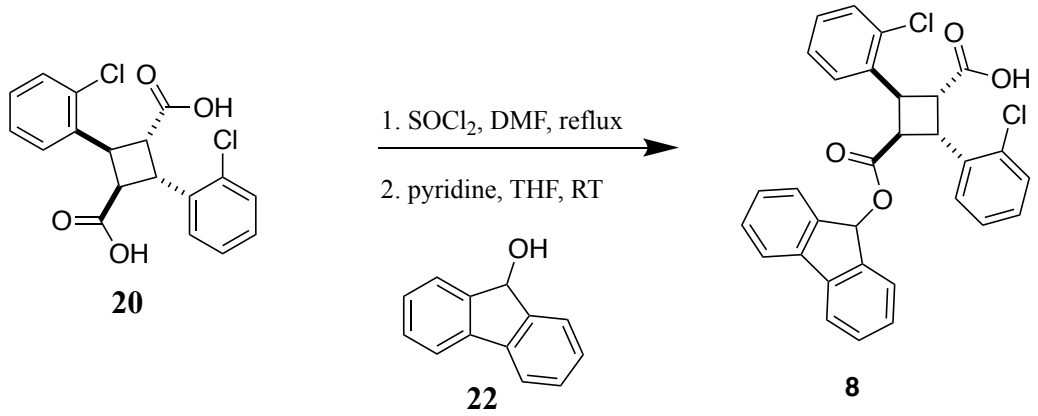
- **Synthesis of (1*S*, 2*S*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)-3-(((2,9-dihydro-1H-fluoren-9-yl)methoxy)carbonyl)cyclobutane-1-carboxylic acid (7)**



(1*R*, 2*R*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)cyclobutane-1,2-dicarboxylic acid **(20)** (165mg, 0.452mmol), thionyl chloride (15mL), and 2-3 drops DMF were added to a 100mL round bottom flask. The reaction mixture was refluxed at 77^{o}C under nitrogen for 3 hours. Toluene was added to the resulting mixture and dried under vacuum. 5mL of THF was added to the resulting residue along with 72.8µL pyridine at 0^{o}C and stirred under nitrogen atmosphere. (9*H*-fluoren-9-yl) methanol **(21)** (84.27 mg, 0.429mmol) was dissolved in 10mL THF and added dropwise to the mixture. The mixture was allowed to gradually warm to room temperature and stirred overnight. The reaction was quenched with water, extracted three times with ethyl acetate, washed with brine and dried with sodium sulfate. The product was dried under vacuum and separated using CombiFlash chromatography with hexanes/ethyl acetate 100:5-100:20. The crude product was dissolved in acetonitrile/methanol and further purified using an Agilent Zorbox SB-C18 reverse phase column on a Shimadzu Prep HPLC. (1*S,* 2*S*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)-3-(((2,9-dihydro-1H-fluoren-9-YL) methoxy) carbonyl) cyclobutane-1-carboxylic acid **(7)** was dried under vacuum and oven dried for 3hrs for a 23.8% yield.

^{1}H NMR (500 MHz, Acetone-d6): δ 7.85 (dd, *J*=7.5, 1.5Hz, 2H), 7.67 (dd, *J*=7.5, 1.5Hz 2H), 7.46 (dd, *J*=7.5, 1.5Hz 2H), 7.42 (t, *J*=7.5, 1.5 Hz, 2H), 7.39 (t, *J*=7.5, 1.5 Hz, 2H), 7.36 (t, *J*=7.5, 1.5 Hz, 2H), 7.24 (m, 2H), 7.16 (m, 2H), 4.98 (dd, *J*=7.0Hz, 2H), 4.43(m, 2H), 4.28 (t, J=7.0Hz, 1H), 4.16 (dd, *J*=7.0Hz, 2H); ^{13}C-NMR (125 MHz, Acetone-d6): δ 172.5, 172.0, 143.1, 141.0, 136.7, 133.4, 129.5, 128.6, 127.3, 126.7, 125.0, 119.8, 52.1, 45.0, 42.2, 38.6, 36.2.

2.4.6 SYNTHESIS OF (1*S*, 2*S*, 3*S*, 4*S*)-3-(((9H-FLUOREN-9-YL)OXY)CARBONYL)-2,4-BIS(2-CHLOROPHENYL)CYCLOBUTANE-1-CARBOXYLIC ACID (8):



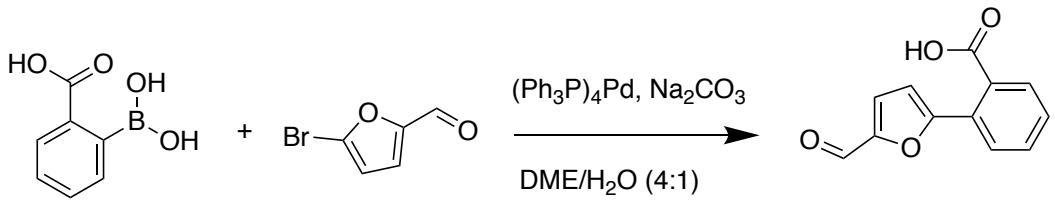
(1*R*, 2*R*, 3*S*, 4*S*)-2,4-bis(2-chlorophenyl)cyclobutane-1,2-dicarboxylic acid **(20)** (85mg, 0.233 mmol), thionyl chloride (3mL), and 2 drops DMF were added to a 100mL round bottom flask. The reaction mixture was refluxed at 77^{o}C under argon for 3 hours. Toluene was added to the resulting mixture and dried under vacuum. 3mL of THF was added to the resulting residue along with 36.86 mg pyridine at 0^{o}C and stirred under nitrogen atmosphere. 9*H*-fluoren-9-ol **(22)** (22mg, 0.116 mmol) was dissolved in 3 mL THF and added dropwise to the reaction. The reaction was allowed to gradually warm to room

temperature and stirred overnight under argon atmosphere. The reaction was quenched with water, extracted three times with ethyl acetate, washed with brine and dried with sodium sulfate. The product was dried under vacuum and separated using CombiFlash chromatography with hexanes/ethyl acetate 100:5-100:20. (1S, 2S, 3S, 4S)-3-(((9H-fluoren-9-yl)oxy)carbonyl)-2,4-bis(2-chlororphenyl)cyclobutane-1-carboxylic acid **(8)** was dried under vacuum and oven dried for 3 hours for a yield of 13.5%.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.64 (dd, *J*=7.5, 1.5, 2H), 7.59 (t, *J*=7.5, 1.5, 2H), 7.39 (q, *J*=7.5, 1.5, 2H), 7.27 (m, 2H), 7.20 (m, 2H), 7.05 (m, 6H), 6.90 (s, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 173, 172.5, 148.8, 141.0, 136.8, 133.4, 129.5, 128.6, 127.3, 126.7, 126.6, 126.2, 126.1, 80.9, 42.8, 38.6.

2.4.7 SYNTHESIS OF (Z)-2-(5-((3-ETHYL-4-OXO-2-THIOXOTHIAZOLIDIN-5-YLIDENE)METHYL)FURAN 2-YL)BENZOIC ACID (9):

- **Synthesis of 2-(5-formylfuran-2-yl)benzoic acid (25)**



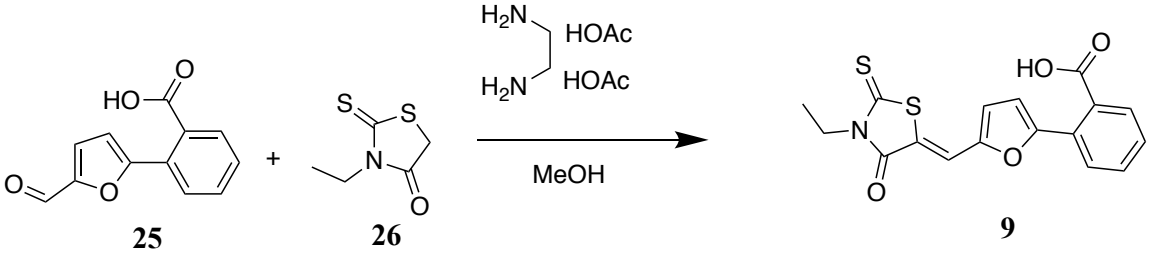
23 **24** **25**

Sodium carbonate (227mg, 2.14mmol), 2-carboxylicpheylboronic acid **(23)** (170mg, 1.03mmol) and 5-bromofuran-2-carbaldehyde **(24)** (150mg, 0.857mmol) were dissolved in 5mL 4:1 DME:H_2O, sonicated, and flushed with argon. 50 mg Palladium-tetrakis(triphenylphosphine) was added to the reaction mixture, sonicated, and flushed

with argon again. The reaction mixture was microwaved for 35 minutes at 100°C. The crude product was extracted with DCM and water and washed with DCM. The pH of the aqueous layer was adjusted to 1 with HCl and extracted with DCM three times. The organic layers were combined, washed with brine, and dried with sodium sulfate. The product was extracted 3 times with dichloromethane, washed with water and brine, and dried with sodium sulfate. 2-(5-Formylfuran-2-yl)benzoic acid **(25)** was dried under vacuum for a yield of 29.2%.

^{1}H NMR (500MHz, $CDCl_3$): δ 9.66 (s, 1H), 7.93 (d, *J*=7.5, 1.5Hz, 1H), 7.71(dd, *J*=7.5, 1.5Hz, 1H), 7.62 (t, *J*=7.5, 1.5Hz, 1H), 7.54 (t, *J*=7.5, 1H), 7.33 (d, *J*=7.5, 1.5Hz, 1H), 6.81 (d, J=7.5 Hz, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 178.0, 167.7, 159.4, 152.3, 134.3, 130.9, 130.8, 128.6, 128.5, 116.2, 109.8.

- **Syntheiss of (Z)-2-(5-((3-ethyl-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan 2-yl)benzoic acid (9)**



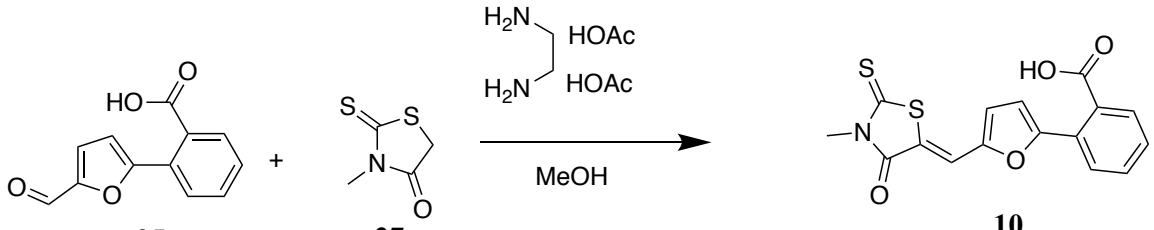
25 **26** **9**

2-(5-Formylfuran-2-yl)benzoic acid **(25)** (25mg, 0.1156mmol), 3-ethylrhodanine **(26)** (18.6mg, 0.1156mmol), 1,2 ethanediamine acetate (20.83mg, 0.1156mmol) and 3mL anhydrous methanol were combined in a round bottom flask, sonicated, and then flushed with argon. The reaction mixture was stirred at room temperature for 24 hrs. The pH of the crude product mixture was adjusted to 1 with 6N HCl and extracted three times with DCM and water. The organic layer was washed twice with brine and dried with sodium

sulfate. (Z)-2-(5-((3-ethyl-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan 2-yl)benzoic acid **(9)** was purified with CombiFlash column chromatography using a 100:0-100:2 gradient of DCM:methanol and dried under vacuum for a 75.1% reaction yield, 21.9% overall yield.

^{1}H NMR (500MHz, $CDCl_3$): δ 7.94 (d, *J*=7.5, 1.5Hz, 1H), 7.71(dd, *J*=7.5, 1.5Hz, 1H), 7.62 (t, *J*=7.5, 1.5Hz, 1H), 7.49 (m, 2H), 6.93 (d, *J*=7.5Hz, 1H), 6.85 (d, , *J*=7.5Hz, 1H), 4.19 (q, *J*=8.0Hz, 2H), 1.25 (t, *J*=8.0Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 189.1, 167.7, 166.1, 156.0, 150.5, 143.0, 134.4, 130.9, 130.8, 128.6, 128.4, 122.0, 121.3, 109.9, 106.4, 41.0, 11.6.

2.4.8 Synthesis of (Z)-2-(5-((3-Methyl-4-oxo-2-Thioxothiazolidin-5-ylidene)Methyl)Furan 2-yl)Benzoic Acid (10):



25 **27** **10**

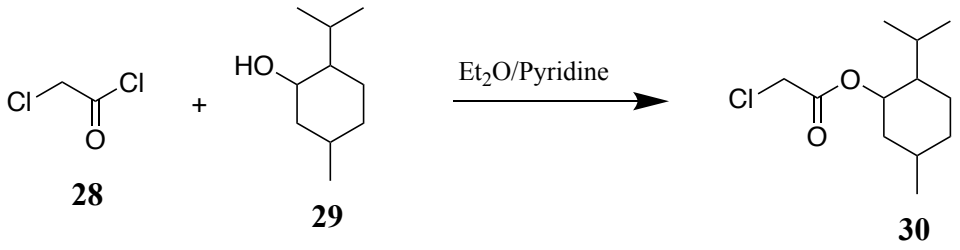
2-(5-Formylfuran-2-yl)benzoic acid **(25)** (29mg, 0.134mmol), 3-methylrhodanine **(27)** (19.75mg, 0.134mmol), 1,2 ethanediamine acetate (24.4mg, 0.134mmol) and 3mL anhydrous methanol were combined in a round bottom flask, sonicated, and then flushed with argon. The reaction mixture was stirred at room temperature for 24 hrs. The pH of the crude product mixture was adjusted to 1 with 6N HCl and extracted three times with DCM and water. The organic layer was washed twice with brine and dried with sodium sulfate. (Z)-2-(5-((3-ethyl-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan 2-yl)benzoic

acid **(10)** was purified with CombiFlash column chromatography using a 100:0-100:2 gradient of DCM:methanol and dried under vacuum for a 99.2% reaction yield, 29% overall yield.

^{1}H-NMR (500MHz, $CDCl_3$): δ 7.96 (d, *J*=7.5, 1.5Hz, 1H), 7.71(dd, *J*=7.5, 1.5Hz, 1H), 7.62 (t, *J*=7.5, 1.5Hz, 1H), 7.50 (m, 2H), 6.95 (d, *J*=7.5Hz, 1H), 6.87 (d, *J*=7.5Hz, 1H), 3.49 (s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 192.6, 167.7, 166.4, 156.0, 150.5, 143.2, 134.4, 130.9, 130.8, 128.6, 128.5, 122.0, 121.3, 109.9, 106.4, 32.0.

2.4.9 SYNTHESIS OF 2-ISOPROPYL-5-METHYLCYCLOHEXYL 2-((4-HYDROXYPYRIMIDIN-2-YL)THIO)ACETATE (11):

- **Synthesis of 2-isopropyl-5-methylcyclohexyl 2-chloroacetate (30)**

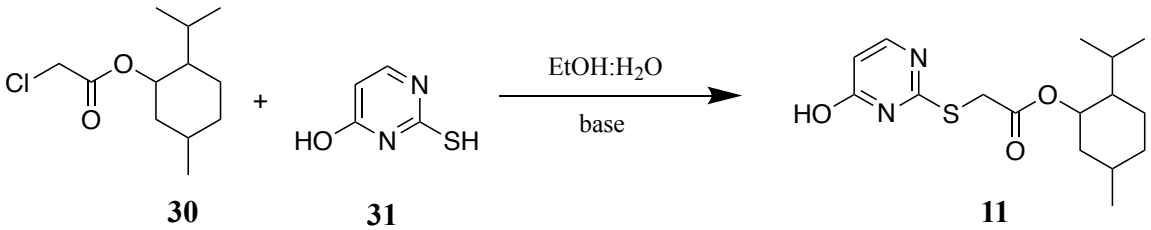


28 **29** **30**

Chloroacetyl chloride **(28)** (0.64mL, 8mmol) was combined with 4mL diethyl ether and added dropwise to a solution of menthol **(29)** (1.25g, 8mmol), 0.65mL pyridine, and 10mL diethyl ether at 0^{o}C under argon. The reaction mixture was stirred at room temperature overnight. The crude product mixture was filtered with 50mL ethyl ether, washed with 6mL 2N HCl followed by 6mL $NaHCO_3$. The product was dried with sodium sulfate and dried under vacuum to yield 2-isopropyl-5-methylcyclohedxyl 2-chloroacetate **(30)** at 91.3%.

^{1}H NMR (500MHz, $CDCl_3$): δ 4.52 (m, 1H), 4.33 (s, 2H), 1.98 (m, 1H), 1.70 (m, 1H), 1.62 (m, 2H), 1.54 (m, 1H), 1.50 (m, 1H), 1.41 (quint, *J*=7.0, 6.8Hz, 1H), 1.38 (m, 2H), 0.83 (dd, *J*=6.8Hz, 6H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 167.0, 74.8, 47.1, 40.8, 40.6, 33.9, 31.4, 26.0, 21.0, 20.7.

- **Synthesis of 2-isopropyl-5-methycyclohexyl 2-((4-hydroxypyrimidin-2-yl)thio) acetate (11)**



30 **31** **11**

2-mercaptopyrimidin-4-ol **(31)** (56.3mg, 0.43mmol), NaOH (19.36mg, 0.48mmol), 1mL EtOH and 1mL H_2O were added to a round bottom flask and heated to 85^{o}C under reflux. 2-isopropyl-5-methylcyclohedxyl 2-chloroacetate **(30)** (115mg, 0.49mmol) was dissolved in 1mL ethanol and added dropwise to the reaction mixture. The reaction kept at 85^{o}C under reflux for 2 hours. The crude product mixture was extracted three times with DCM and water and washed three times with brine. The product was dried with sodium sulfate and purified using CombiFlash column chromatography with a 100:0-100:32 hexanes: ethyl acetate solvent gradient. Pure 2-isopropyl-5-methycyclohexyl 2-((4-hydroxypyrimidin-2-yl)thio) acetate **(11)** was recovered at 55.6% reaction yield, 50.8% yield overall.

^{1}H NMR (500MHz, $CDCl_3$): δ 7.82 (d, *J*=7.5Hz, 1H), 6.28 (dd, *J*=7.5Hz, 1H), 4.74 (td, *J*=7.0Hz, 1H), 3.92 (s, 2H), 1.98 (m, 1H), 1.85 (m, 1H), 1.67 (m, 2H), 1.45 (m, 2H), 1.41 (quint, *J*=7.0, 6.8Hz, 1H), 1.03 (m, 7H), 0.83 (dd, *J*=6.8Hz, 6H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 169.4, 167.0, 163.7, 153.6, 111.2, 74.8, 47.1, 40.8, 36.1, 33.9, 31.4, 26.0, 23.9, 21.0, 20.7.

2.4.10 TRANSACTIVATION REPORTER ASSAY

Cos7 cells were cultured in charcoal treated media and co-transfected with vectors encoding a luciferase reporter driven by three copies of peroxisome proliferator response element (PPRE), an expression vector for PPARδ, and an expression vector β-galactosidase as a transfection control. Transfected cells were cultured in serum-free media for 4 hrs. and then treated with serum-free media containing 1μM PPARδ agonist GW501516 or 20μM of the compound of interest for 18hrs. Following incubation, cells were lysed with 1xReporter Lysis Buffer. β-galactosidase activity was measured by incubated 30μL cell lysate with 100xMg solution (0.1M $MgCl_2$, 4.5M β-mercaptoethanol), 0.1M sodium phosphate (pH 7.5), and 1XONPG (4mg/mL o-nitrophenyl-β-D-galactopyranoside in sodium phosphate buffer) until a strong yellow color developed. Absorbance was read at 420nM. Luciferase activity of the lysates was measured using the luciferase reporter assay system and normalized to β-galactosidase activity. Values are calculated as a fold change relative to untreated cells.

2.4.11 PROLIFERATION ASSAYS

Cells were seeded at 5,000 cells/well in a 96-well plate and allowed to adhere overnight. Media containing the compound of interest was applied to the cells and changed every other day for 4 days. On the fourth day, PrestoBlue Cell Viability reagent was added to the cells and incubated for 30-60min. Fluorescence was read at λ_{ex}=560nM/λ_{em}=590nM. Fold proliferation is calculated relative to the highest fluorescence intensity among untreated cells.

2.4.12 FABP5 KNOCKDOWN

293T cells were co-transfected with p.LKO.1 vectors encoding either control shRNA or a non-functional GFP-coding shRNA and an shRNA targeting FABP5 using Polyfect. The lentiviruses were produced using pCMV packaging and pMD2.G envelope vectors. The viruses were applied to MDA-MB-231 cells and successful infection was selected using puromycin.

2.4.13 qRT-PCR

100mm plates of cells at 80-90% confluency were incubated with compound for 6hrs at 37^{o}C and 5% CO_2. Following incubation, total RNA was extracted using TRIZol reagent or Qiagen RNeasy Mini Kit. RNA purity was evaluated via UV-Vis NanoDrop spectrophotometry. cDNA was synthesized from RNA using an iScript cDNA synthesis kit and used in subsequent reactions at a 1:5 dilution. qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System or BioRad CFX96 Touch Real Time PCR Detection System with TaqMan assay probes and TaqMan Fast Advanced

Master Mix. Fold change in gene expression is calculated relative to expression levels of transcription control and the target gene in untreated cells.

2.5 ACKNOWLEDGEMENTS

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CHAPTER 3. THE STRUCTURE-ACTIVITY RELATIONSHIP OF COMPOUND 6 AND FABP5

3.1 INTRODUCTION

The relationship of structure and function is one of the guiding principles of biochemistry and a cornerstone of drug design, especially when target specificity is a concern. The complex relationship between target and ligand means that even simple changes to a drugs' geometry can dramatically change the activity and efficacy of that compound. For example, cisplatin, a chemotherapeutic that works by interfering with DNA replication, is only pharmacologically active in its cis conformation.[1] The trans conformation of the compound, transplatin, is clinically inactive due to its increased reactivity and reduced ability to form the intra-strand adducts that allow cisplatin to interfere with DNA replication.[2] In the case of FABP5, the tertiary nuclear localization sequence (NLS) contained within the alpha-helical cap of FABP5 is inactivated if L-shaped unsaturated fatty-acids bind the protein and prevent its proper formation while U-shaped saturated fatty-acids tend to fit comfortably into the binding pocket and allow the NLS to properly form.[3] The wide range of potential FABP5 ligands and diverse functions means that understanding the structure-activity relationship of any potential inhibitor is tantamount to fully understanding the extent and nature of protein inactivation rendered.[4–7]

Compound **6** is a commercially available potential FABP5 inhibitor capable of reducing activation of PPARδ and decreasing proliferation in FABP5-rich MDA-MB-231 cells (Figure 3.1). While the manufacturer of compound **6** provides a basic structure of the inhibitor, there are eight possible isomers of compound **6** (Figure 3.1). Four isomers are "endo" at carbons 2 and 3 while the remaining four are "exo" at carbons 2 and 3.

Among each set of "endo" or "exo" compounds, carbons 2 and 3 may be R or S, depending on the regioisomer. Each stereoisomer may be further divided based on the chirality of carbon 9. The manufacturer of compound **6** specifies the structure as 2R, 3S but provides no further information regarding endo or exo conformation or the chirality of carbon 9. This complicated structure specificity further necessitates a deeper understanding of the structure-activity relationship of compound 6 and FABP5.

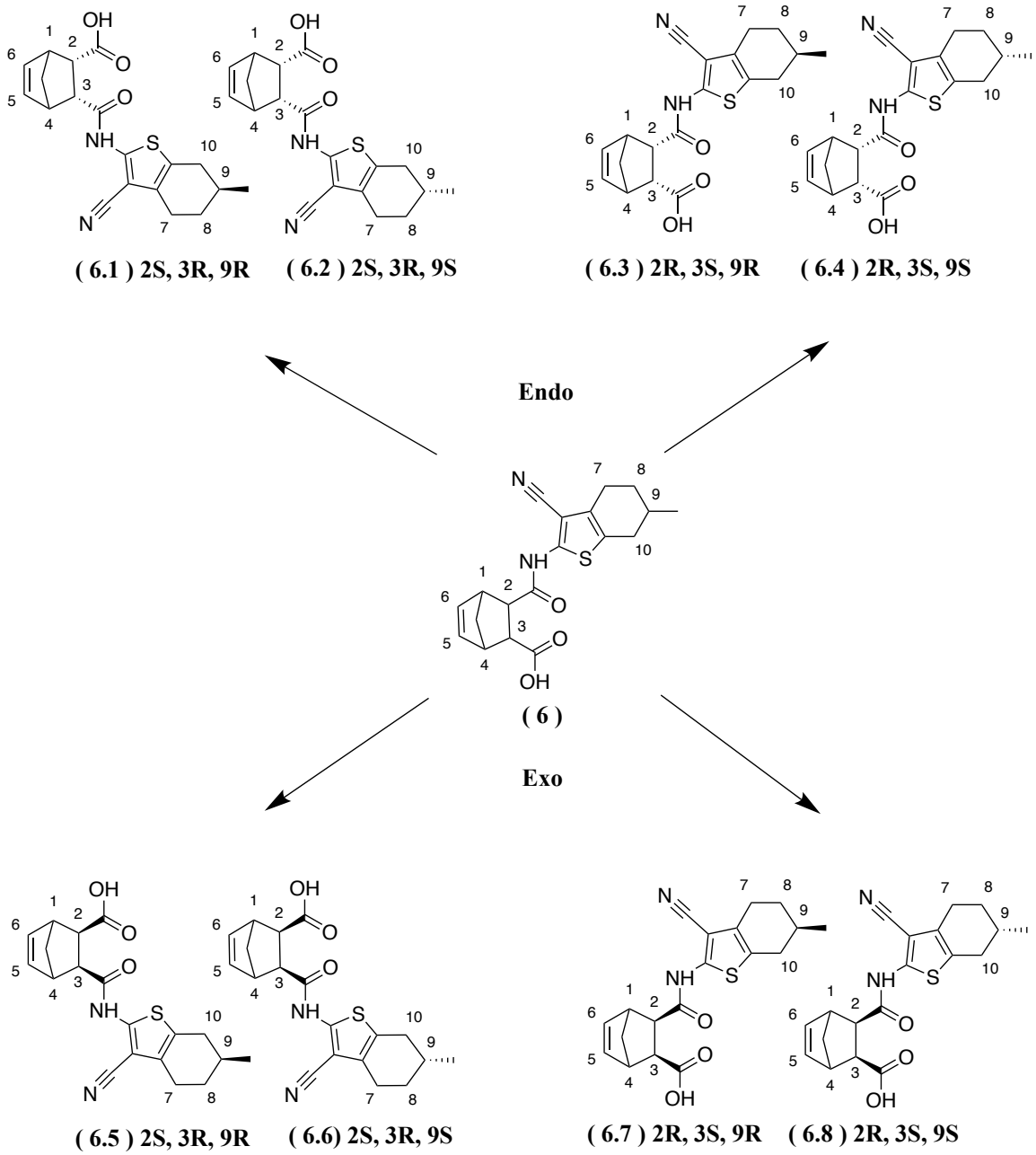


Figure 3.1 Possible Regioisomers and Stereoisomers of Compound 6.

3.2 Results and Discussion

3.2.1 Structural Determination of Compound 6 and Its Isomers

An ^{1}H-NMR spectrum of compound **6** in deuterated chloroform shows easily identifiable signals for hydrogens attached to carbons 1-6, but the region that contains the signals for carbons 7-10 is muddled with poor signal resolution (Figure 3.2a). When the ^{1}H-NMR spectrum is taken in deuterated methanol, however, the resolution is vastly improved and each hydrogen signal is easily assigned (Figure 3.2b). Increased signal resolution in a higher-polarity solvent like methanol indicates that there is increased hydrogen bonding between molecule and solvent and less intra-hydrogen bonding between molecules, suggesting that compound **6** forms a dimer in certain solvents of limited polarity.[8,9]

Importantly, the J-coupling between hydrogens 1 and 4 and hydrogens 2 and 3 is around 3.3Hz, indicating that hydrogens 2 and 3 are in the endo position relative to hydrogens 1 and 4 (Figure 3.2b).[10,11] Furthermore, the NOESY spectrum of compound **6** in methanol shows a clear through-space interaction between the bridge hydrogens, hydrogens 1 and 4, and hydrogens 2 and 3 (Figure 3.3). This means that compound **6** is likely endo at carbons 2 and 3.

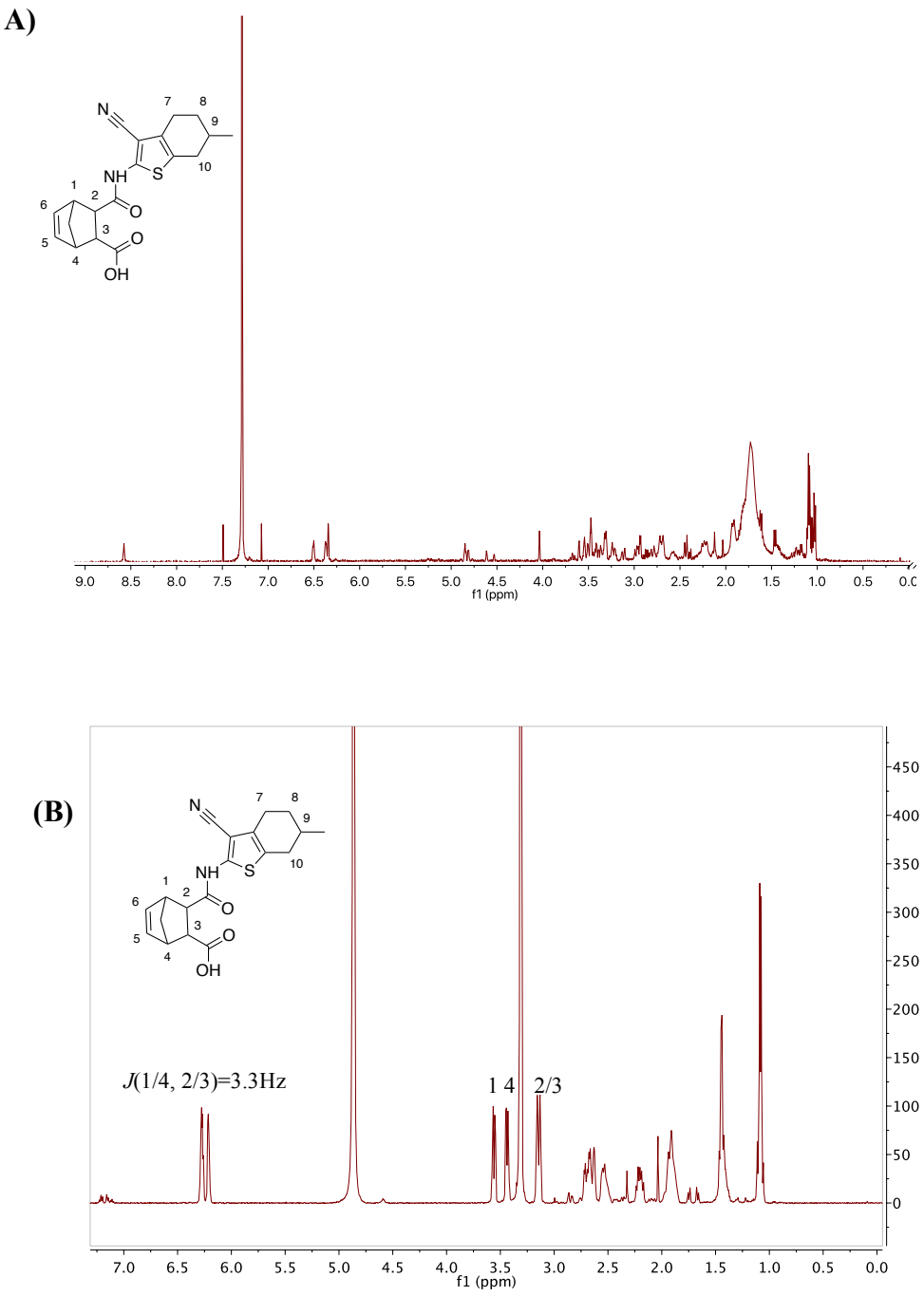


Figure 3.2 ^{1}H-NMR of Compound 6 in $CDCl_3$ (a) and MeOD (b). The increased resolution in MeOD indicates the formation of a dimer in less polar solvents. The large coupling constant between hydrogens 1 and 4 and 2 and 3 (J=3.3Hz) is indicative of endo stereochemistry.

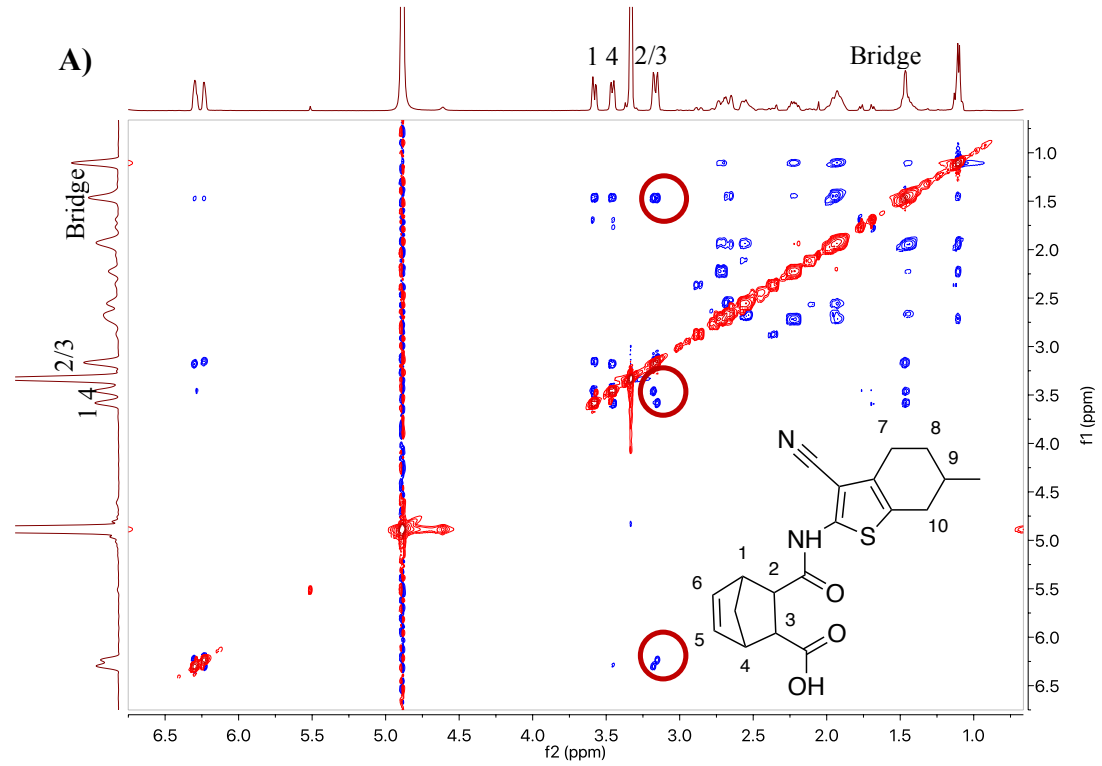
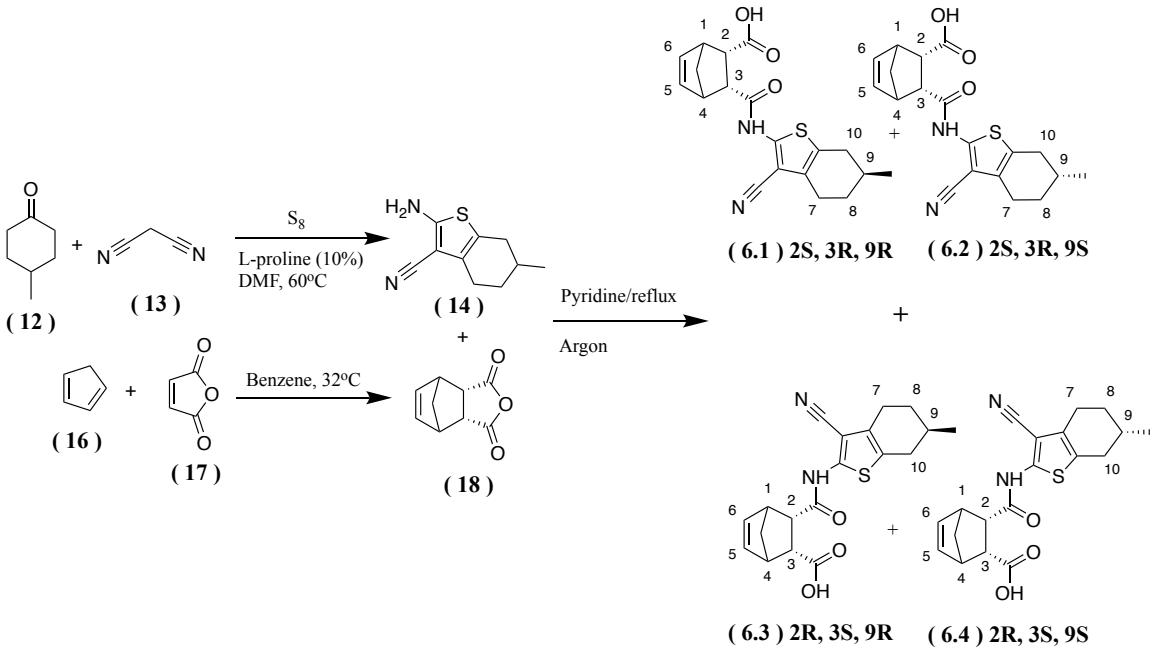
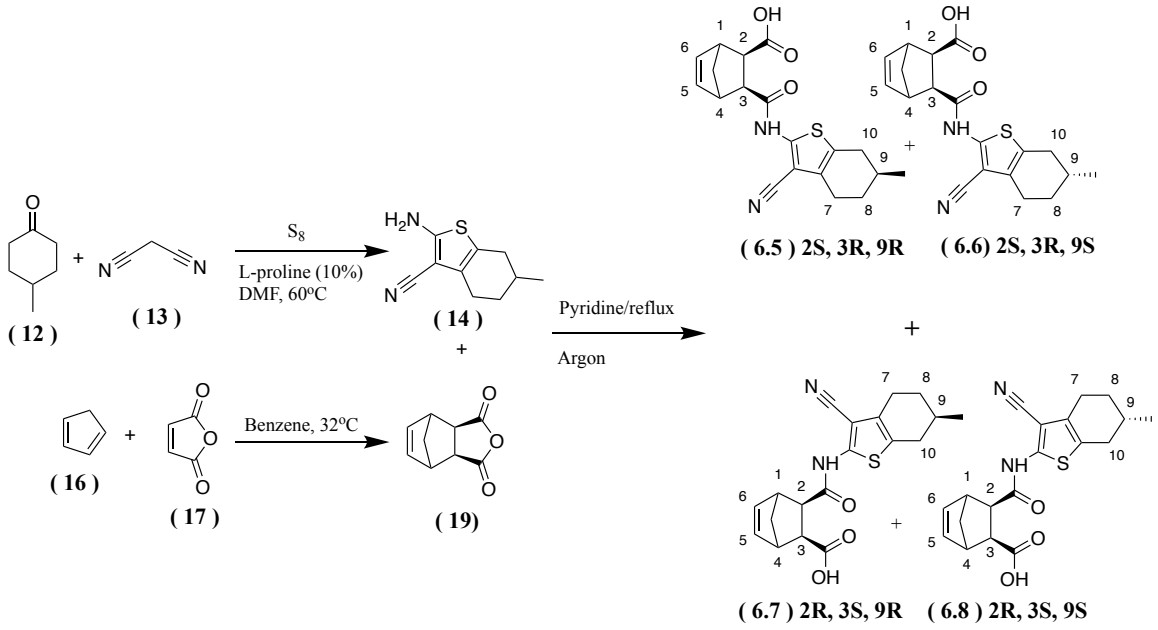


Figure 3.3 (A) NOESY spectrum of compound 6 and graphic (B) showing NOE interactions, in red, indicated on NOESY spectrum. There is a through-space interaction between hydrogens 2 and 3, hydrogens 1 and 4, and the bridge hydrogens. This provides evidence that commercial compound 6 is in the endo conformation.

While the manufacturer reports the stereochemistry of compound **6** as 2R, 3S, this claim must be verified experimentally. In order to elucidate the stereochemistry of carbons 2, 3 and 9 on commercially available compound **6,** Yuzhi Shang, Yonghung Hou and Emma Whiting synthesized each possible isomer of compound **6** as a racemic mixture. Each synthesis followed the same route: a Gewald reaction to form an amine intermediate (**14)**, a Diels-Alder reaction to form a mixture of endo- (**18**) and exo-anhydride (**19**). Intermediates (**14)** and purified (**18)** or (**19)** were then reacted to form the amide bond, yielding compounds **6.1**-**6.8** as racemic mixtures (Scheme 3.1 and 3.2).



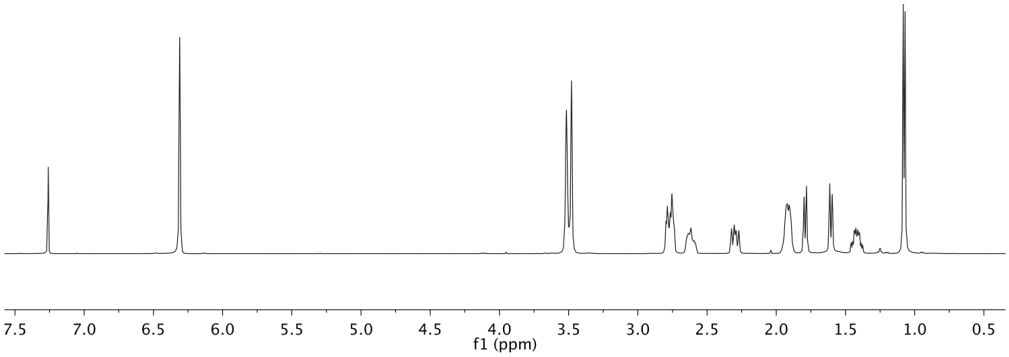
Scheme 3.1 Synthesis of Endo-Isomers of Compound 6.



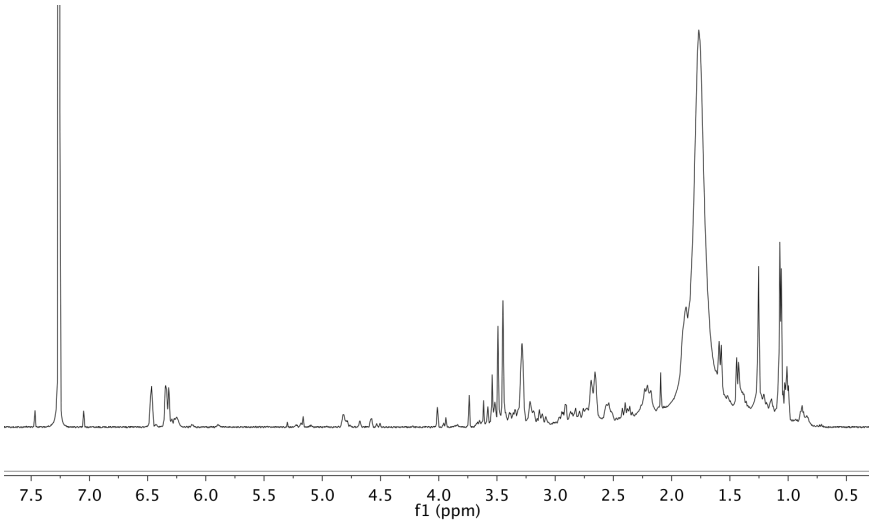
Scheme 3.2 Synthesis of Exo-Isomers of Compound 6.

Unlike compound **6**, the mixture of endo-isomers **6.1+6.2** displays a clear and identifiable ^{1}H-NMR spectrum in both $CDCl_3$ and MeOD, suggesting that this mixture does not form a dimer and adopts the 2S, 3R regioisomer (Figure 3.4a). The mixture of **6.3+6.4**, however, does display muddled ^{1}H-NMR resolution in $CDCl_3$ that is eliminated with the increased polarity of MeOD, suggesting that **6.3+6.4** adopt the 2R, 3S conformation (Figure 3.4b and c). Together, these NMR data indicate that compound **6** is endo at carbons 2 and 3 and is the 2R, 3S stereoisomer.

A)



B)



C)

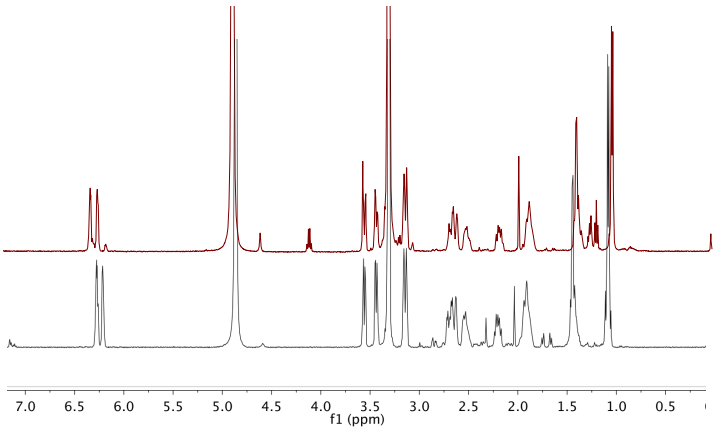


Figure 3.4 Proton NMR spectra for compound 6 isomers in $CDCl_3$ and MeOD. A)^{1}H-NMR spectrum of **6.1**+**6.2** in $CDCl_3$ displays a clear and definable spectrum. B) ^{1}H-NMR spectrum of **6.3**+**6.4** in CDCL3 is muddled while the same compound in MeOD, shown in red, (C) is clear and definable, similar to commercial compound **6**, shown in black.

In order to verify the endo, 2R, 3S isomer and in order to elucidate he chirality of carbon 9, a single crystal XRD analysis of the structure of commercial compound **6** was completed by Heather Folkwein-Kennehan. An initial resolved XRD structure confirms a 2R, 3S conformation for the commercial compound (Figure 3.5). While the crystal structure does confirm the chirality of carbons 2 and 3, it does display a slightly different structure with a second five-membered ring forming between the carbonyl and amine groups. This change in structure is likely due to solvent evaporation effects, but it does necessitate another future single crystal analysis in order to confirm the reported structure of compound **6**. Interestingly, the crystal structure indicates that the compound exists as a mixture of isomers with carbon 9 at about 70% R, 30% S.

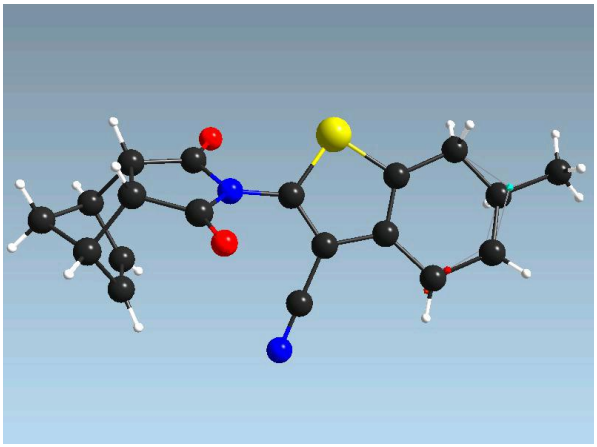
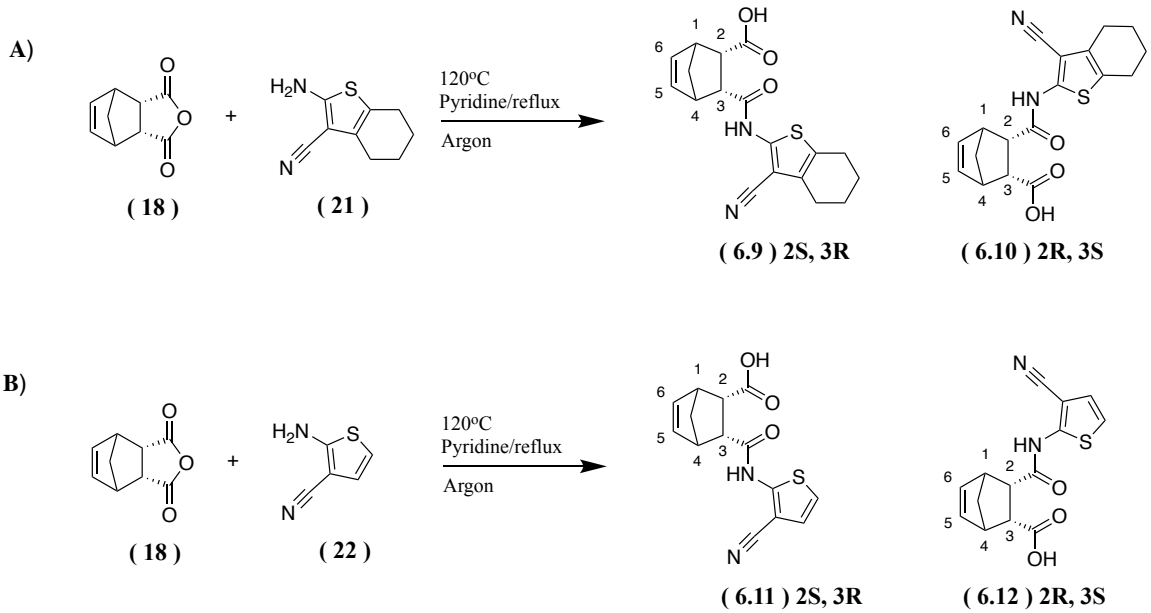


Figure 3.5 Preliminary XRD crystal structure of commercial compound **6**. The five-membered ring displayed between the carbonyl and amine groups is not reported in the original structure and likely forms due to solvent evaporation during crystal formation.

Given this data, compound **6** is indeed endo at carbons 2 and 3 and is likely in the 2R, 3S conformation. However, the exo isomers are necessary to fully examine the structure activity relationship of the compound. Among the exo isomers of compound **6**,

the racemic mixture of **6.5**+**6.6** elutes before the other exo regioisomers, just as **6.1**+**6.2** eluted first. This is a strong indication that **6.5**+**6.6** are in the 2S,3R conformation. Interestingly, the ^{1}H-NMR spectrum of **6.5**+**6.6** in $CDCl_3$ has clear resolution but becomes muddled in MeOD, a change which indicates aggregation in higher polarity solvents. This is in direct contrast to its endo counterpart, **6.1**+**6.2**, which aggregates in less polar solvents. This change in hydrogen-bonding behavior between endo and exo isomers suggests that there may be some significant differences in the actual inhibitory activity of the mixtures. The exo 2R, 3S mixture, **6.7**+**6.8,** has muddled resolution in $CDCl_3$, indicating a similar hydrogen-bonding activity as its endo counterpart, **6.3**+**6.4**.

In addition to exploring the efficacy of the different compound **6** isomers, Yuzhi Shang and Yonghung Hou synthesized an endo 2S, 3R and 2R, 3S derivative without the methyl group on carbon 9, **6.9** and **6.10,** as well as endo 2S, 3R and 2R,3S derivatives with no cyclohexane attached to the thiophene, **6.11** and **6.12** (Scheme 3.3). Data from these derivatives will determine if the methyl group or cyclohexane ring are essential to the functionality of compound **6**.



Scheme 3.3 Synthesis of compound 6 derivatives with no methyl group, 6.9 and 6.10 (a) and no cyclohexane ring (b), 6.11 and 6.12.

3.2.2 Evaluation of Inhibitor Efficacy

In order to best evaluate the structure activity relationship of compound **6** and FABP5, each isomer is first evaluated on its individual ability to inhibit FABP5. Like the parent compound **6**, each isomer must meet two criteria in order to be an effective candidate inhibitor: it cannot independently activate PPARδ and it must bind FABP5 in such way that reduces PPARδ-mediated cell proliferation. In order to ensure effective FABP5 inhibition, each isomer mixture was tested for its ability to activate PPARδ in a PPRE transactivation assay. Cos7 cells were co-transfected with expression vectors for a luciferase-linked PPRE reporter and the PPARδ protein and then evaluated for luciferase activity following incubation with each isomer for 18hrs. As with the parent compound **6**, there is a slight elevation in luciferase with each racemic mixture but none of the isomers or derivatives induce any significant PPARδ activation (Figure 3.6).

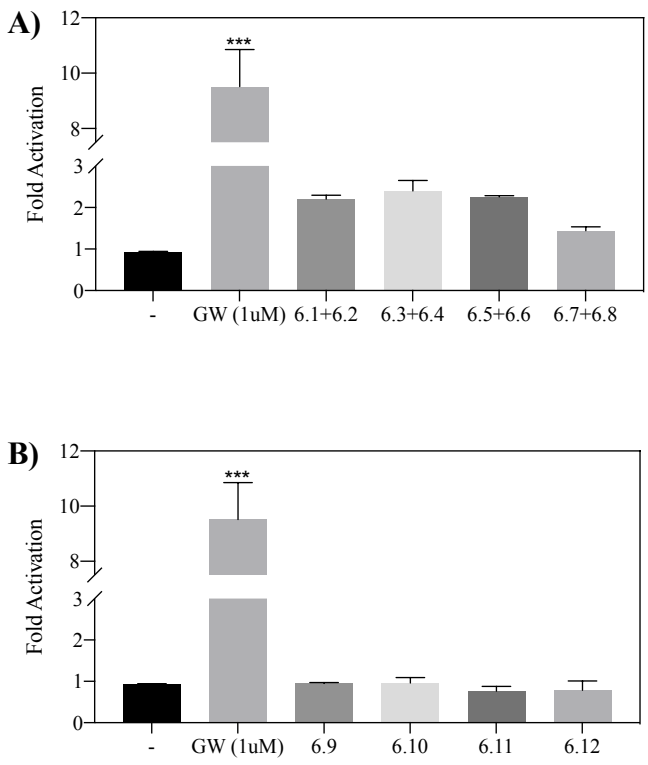


Figure 3.6. PPRE transactivation assays of (A) each compound 6 isomer and (B) each compound 6 derivative. While there is a slight elevation in luciferase activity, no isomer induces significant activation of PPARδ. There is no PPARδ activity after treatment with any compound 6 derivative. n=2, ***p=0.0002

To evaluate the ability of these derivatives to hinder cell proliferation via FABP5 inhibition, MDA-MB-231 cells were incubated with 5, 10, or 20μM of each compound for four days. Cell viability was then measured with PrestoBlue Cell Viability reagent. Only **6.7**+**6.8**, the exo-2R, 3S, 9R/S mixture, is unable to induce any decrease in proliferation (Figure 3.7a).

Interestingly, while the NMR characterization data suggest that commercial compound **6** and the **6.3**+**6.4** mixture share a structure and display similar self-aggregating behavior in non-polar solvents, the synthesized mixture was unable to replicate the same reduction in proliferation seen with the commercial compound.

Meanwhile the anti-proliferative activity of the endo-2S, 3R mixture, **6.1**+**6.2**, produces a reduction in anti-proliferative activity nearly identical to that of the commercial parent compound at 20μM, despite their structural differences (Figure 3.7a). This suggests that either the chirality of carbon 9 or additional factors outside of the chirality of carbons 2 and 3 direct the compound **6** inhibitory activity.

When 231 cells are incubated with endo derivatives lacking the methyl group, **6.9** and **6.10**, the decrease in proliferation is diminished to insignificance and is no longer dose-responsive (Figure 3.7b). There is little difference in anti-proliferative activity between the two isomers, suggesting that the methyl group may have more bearing on inhibitory activity than the chirality of carbons 2 and 3. The decrease in proliferation disappears altogether when the cyclohexane group is removed. This indicates that the cyclohexane group is essential to the inhibitory activity of compound **6** and that the methyl group likely plays a key role in inhibitor efficacy.

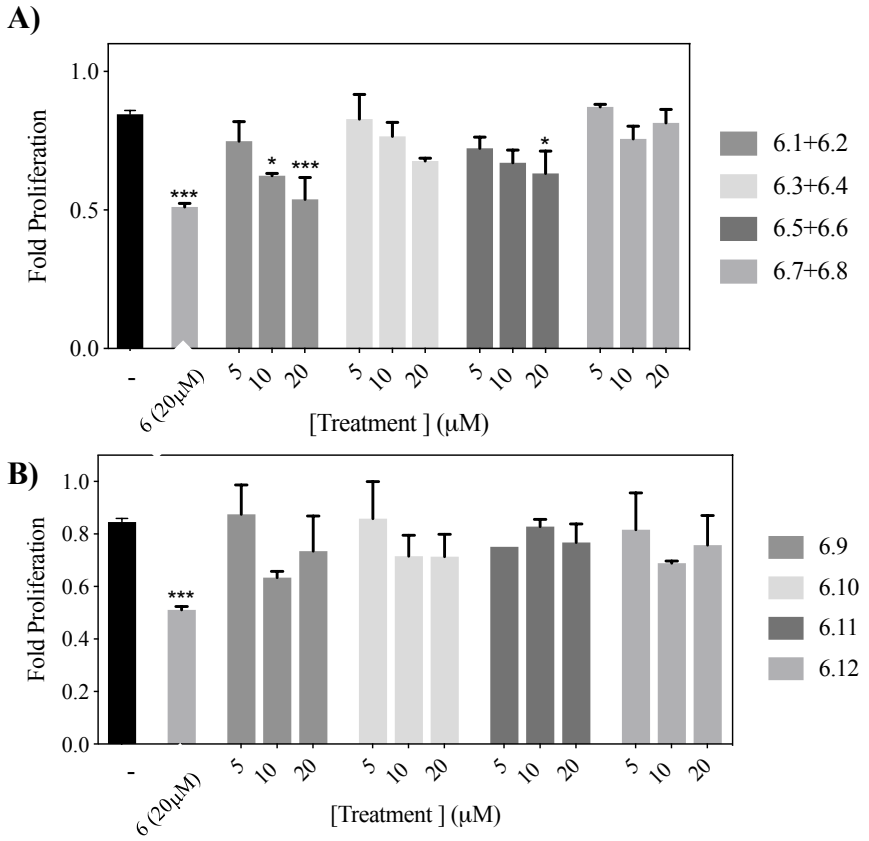


Figure 3.7. Proliferation assays in MDA-MB-231 cells treated with racemic mixtures of compound 6 isomers (A) and derivatives (B). Only the 2S, 3R mixtures induced any significant reduction in cell proliferation. Eliminating the methyl or cyclohexane groups eliminates inhibitory activity n=2, *p=0.02, ***p=0.002.

In addition to their effects on cell proliferation, each active compound **6** isomer must be evaluated for their effects on the expression of PPARδ and RAR target genes. If FABP5 is effectively inhibited, the expression of PPARδ targets genes should decrease while RAR target genes should increase. When MDA-MB-231 cells are incubated with the endo compound **6** isomers, the expression of PPARδ target genes *PLIN2* and *CD47* is significantly reduced after incubation with **6.1+6.2** (Figure 3.8a). This is in good agreement with the reduced cell proliferation seen after incubation with the endo 2S, 3R

mixture. This same mixture does not induce any real change in RAR target gene expression (Figure 3.8b). This may be due to the relatively low expression of RAR signaling machinery in estrogen receptor negative breast carcinoma cell lines such as MDA-MB-231.[12] Interestingly, **6.3+6.4**, the isomer mixture that bears the closest resemblance to commercial compound **6**, induces a slight increase in PPARδ target gene expression, but this change is not significant. There is, however, a noticeable increase in the expression of RAR target genes after incubation with this mixture. This likely accounts for the modest decrease in proliferation seen with **6.3+6.4** treatment. Treatment with the **6.5+6.6** mixture decreases expression of both PPARδ and RAR target genes. Together with the effects on cell proliferation, these data suggest that while the structure of compound **6** most closely resembles that of **6.3+6.4**, that structure similarity does not translate to similar effects in cell proliferation and gene expression. In fact, the *2S,3R* endo isomer mixture **6.1+6.2** induces better anti-proliferative results.

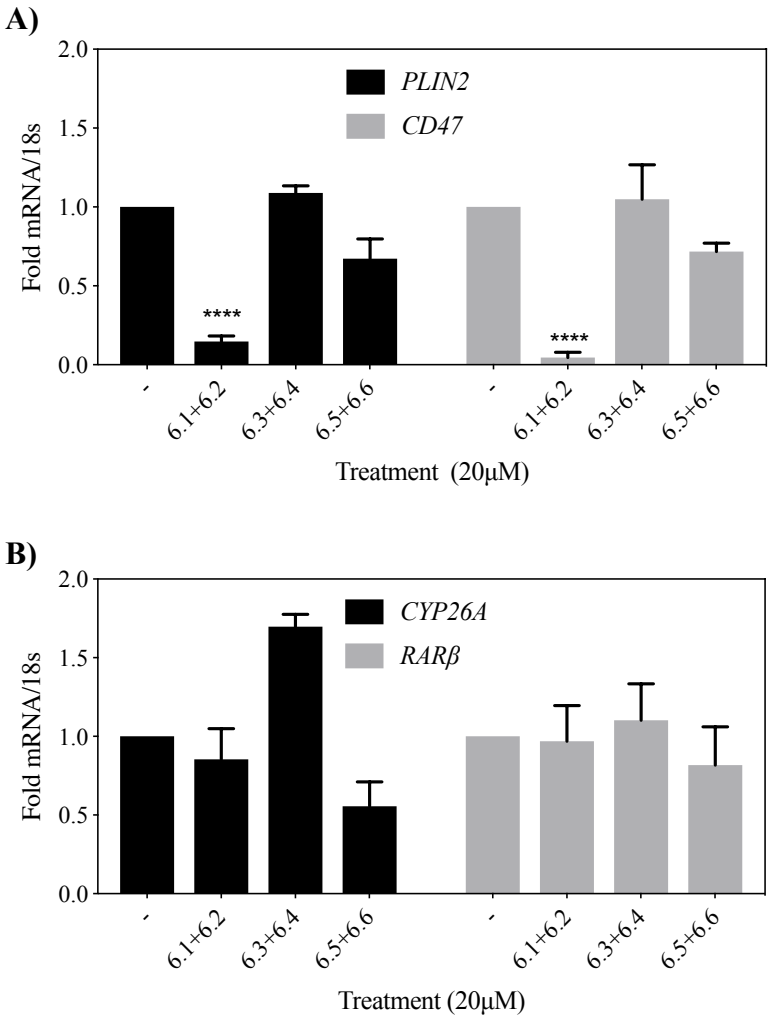


Figure 3.8. Effect of compound 6 derivatives on PPARδ (A) and RAR (B) target genes in MDA-MB-231 cells. Incubation with endo 2S, 3R mixture **6.1+6.2** leads to the most significant reduction in PPARδ. The effect on RAR target genes is less pronounced due to reduced RAR signaling endogenous in MDA-MB-231 cells.

Interestingly, the ^{1}H-NMR spectrum of compound **6** in MeOD displays some impurities that correlate to peaks seen in the MeOD ^{1}H-NMR spectrum of the **6.1+6.2** mixture (Figure 3.9). When the peaks are integrated, it appears that commercial compound **6** is actually a mixture of the **6.1+6.2** and **6.3**+**6.4** isomers in an approximate 1:9 ratio. This suggests that the activity of compound **6** may rely on a mixture of the **6.1+6.2** and **6.3**+**6.4** isomers rather than a mixture that is purely *2S, 3R* or *2R, 3S*.

In order to test this hypothesis, 231 cells were incubated for four days with 5, 10, or 20μM of varying ratios of **6.1+6.2** and **6.3**+**6.4** isomers (Figure 3.10) and cell proliferation was evaluated using PrestoBlue Cell Viability reagent. While there is an observed dose-responsive decrease in proliferation, the same 9.36:1 ratio of synthesized **6.3**+**6.4**:**6.1+6.2** is unable to replicate the decrease in cell proliferation observed with commercial compound **6**. However, as the **6.3**+**6.4**:**6.1+6.2** ratio is decreased, the dose-responsive decrease in cell proliferation becomes stronger and more significant, with the most promising dose-responsive pattern observed at 1:2 **6.3**+**6.4**:**6.1+6.2.** The proliferation fell to about 19% at 20μM treatment with this mixture. The fold proliferation at 20μM is only 53% for **6.1+6.2** alone and 67% for **6.3**+**6.4,** providing a strong indication that while not observed in the original commercial composition, a 1:2 ratio of the two endo isomers of compound **6** may improve inhibitor performance.

When MDA-MB-231 cells are incubated with the 1:2 **6.3**+**6.4**:**6.1+6.2** isomer mixture, there is no significant change in either PPARδ or RAR target gene expression (Figure 3.11). There is a simultaneous increase in RAR gene expression with a slight decrease in PPARδ gene expression, but these changes are not significant and quite similar to those observed with **6.3**+**6.4** alone. This stands in contrast to cells treated with **6.1**+**6.2** alone, which induces a very significant decrease in both *Plin2* and *CD47* (Figure 3.8 and 3.11). Notably, the isomer mixture does induce a slight increase in RAR target gene expression while those genes remain essentially unchanged with **6.1**+**6.2**. This modest change in both PPARδ and RAR gene expression does not account for the significant reduction in MDA-MB-231 cell proliferation seen with the 1:2 isomer mixture when compared to either isomer alone (Figure 3.10 and 3.7). This is an indication that the

1:2 isomer mixture may be working outside of the PPARδ/FABP5 signaling pathways to induce anti-proliferative effects.

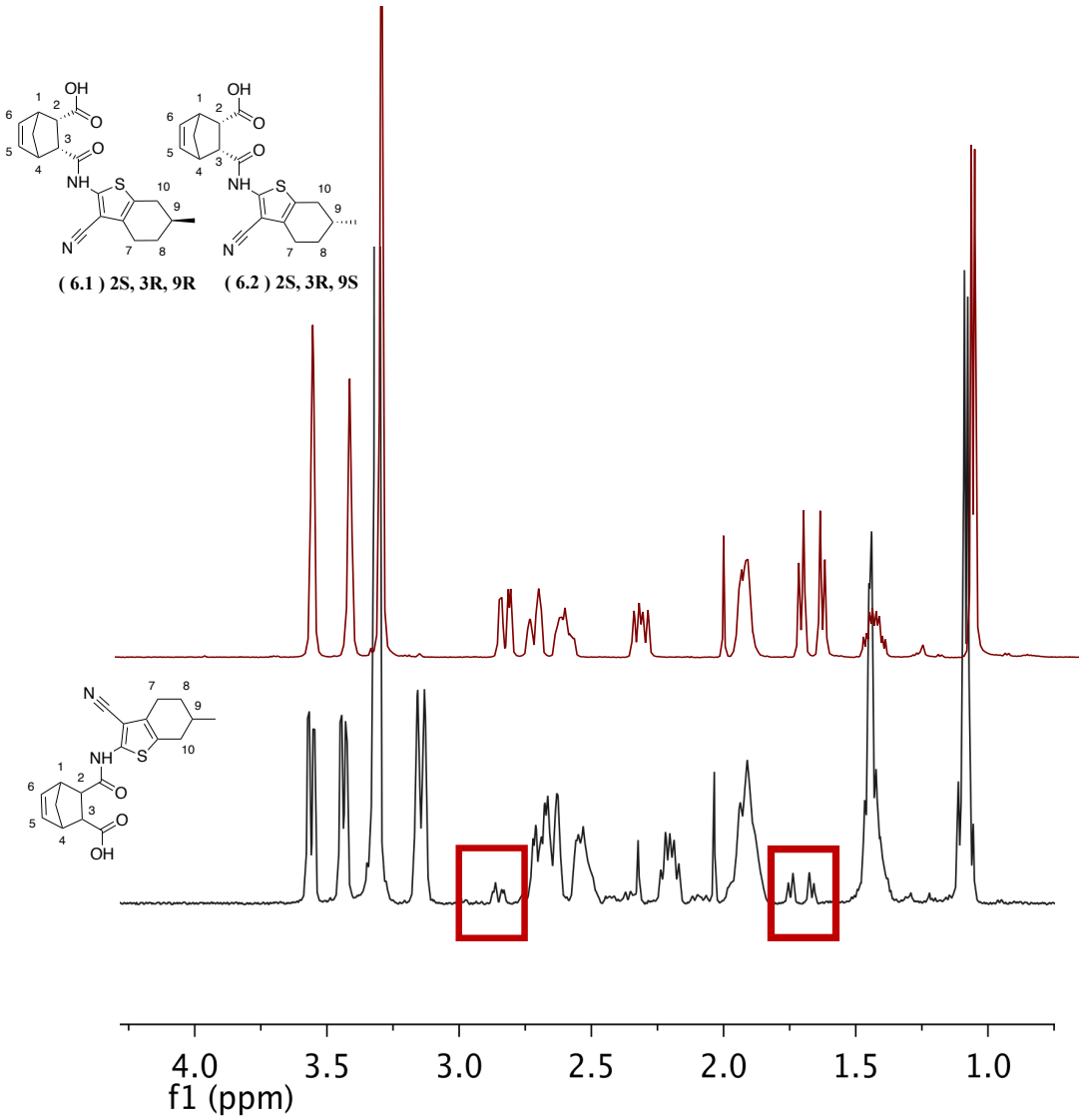


Figure 3.9. ^{1}H-NMR spectra in MeOD of the **6.1**+**6.2** endo isomer mixture, shown in red, and commercial compound **6**, shown in black. The spectrum of compound **6** shows small impurities that correlate to signals seen in the spectrum of the **6.1**+**6.2** mixture. This means that compound **6** is likely a mixture of **6.1**+**6.2** and **6.3**+**6.4** in an approximate 1:9 ratio.

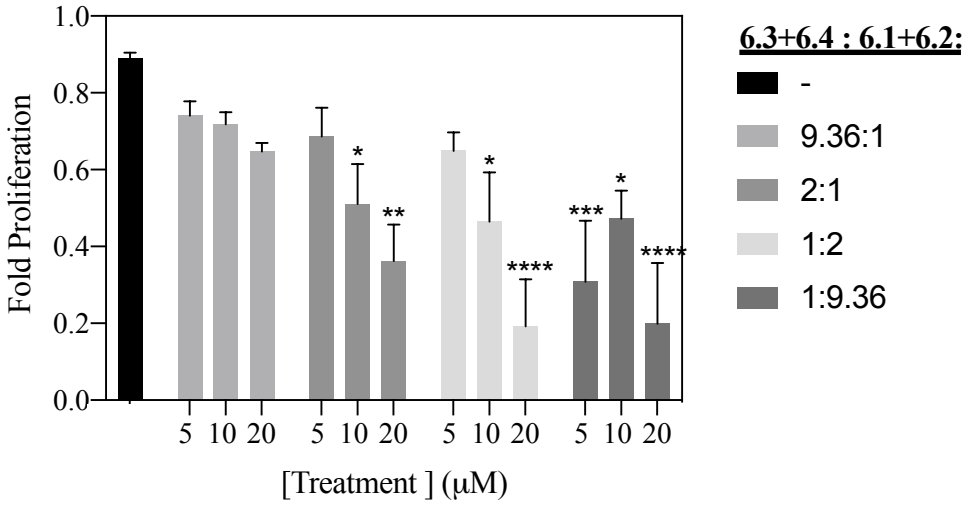


Figure 3.10 Proliferation of MDA-MB-231 cells incubated with different ratios of the **6.3**+**6.4** and **6.1**+**6.2** isomer mixtures for four days. As the ratio of **6.3**+**6.4**:**6.1+6.2** decreases, the observed decrease in proliferation increases, suggesting a key role for the **6.1**+**6.2** mixture in FABP5 inhibition. n=3 **info on significance**

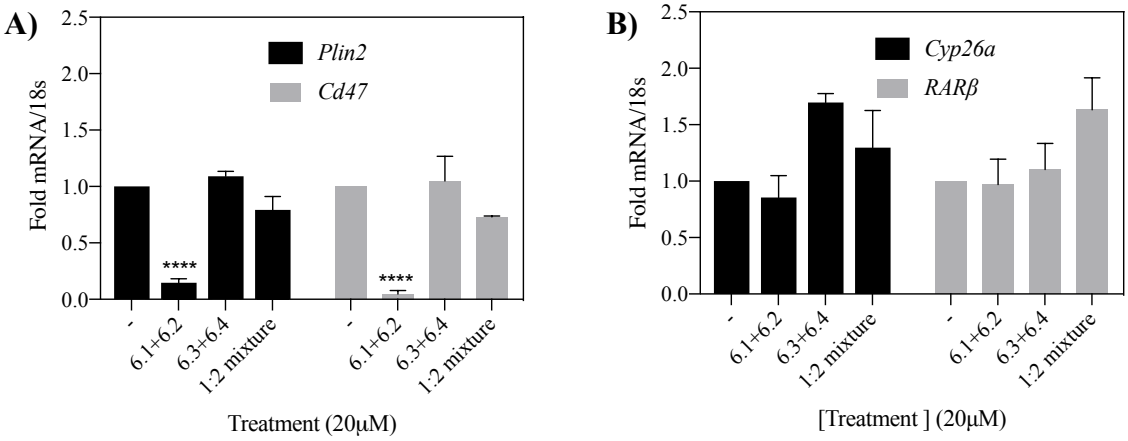


Figure 3.11 PPARδ (A) and RAR (B) target gene expression in MDA-MB-231 cells after incubation with 20μM of **6.1+6.2**, **6.3+6.4** or the 1:2 **6.3**+**6.4**:**6.1+6.2** isomer mixture. While there is a slight decrease in PPARδ gene expression and an increase in RAR gene expression after incubation with the isomer mixture, these changes are not significant. n=3, ****p<0.0001

One possible explanation of this improved inhibitor performance with the isomer mixture is an increased activation of other PPAR proteins. When PPARα, δ, and γ activation after incubation with compound **6** or the 1:2 isomer mixture is evaluated by transactivation assay, neither compound induces any significant activation of PPARδ or γ (Figure 3.12). However, both compounds are able to induce significant activation of PPARα, with the isomer mixture inducing a significantly higher activation than that rendered by compound **6** alone. Increased activation of PPARα by arachidonic acid has been shown to induce an increase in MDA-MB-231 cell proliferation, but several other studies point to increased PPARα activity as anti-proliferative. Evidence suggests that the response to PPARα activation largely depends on tumor type and environment.[13–16] Future experiments should examine the effect of isomer mixture treatment on the expression of PPARα target genes as well as downstream effects on PPARα-mediated lipogenesis. It should also be examined if these effects are mediated by FABP5 or some other FABP or if the mixture acts independently of any intracellular binding proteins.

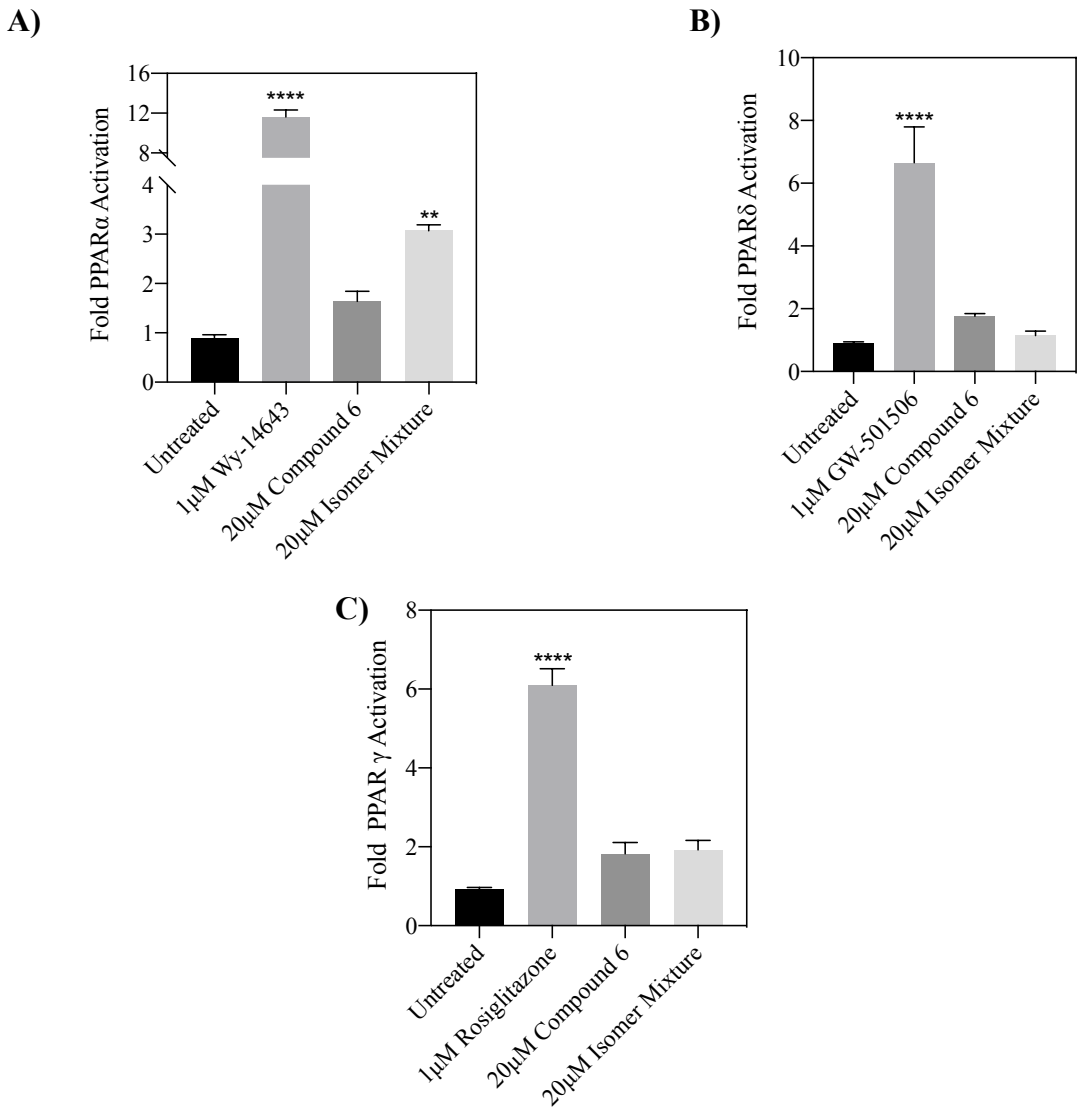


Figure 3.12. Transactivation assays of PPARα (A), PPARδ (B) and PPARγ (C) with compound **6** and a 1:2 mixture of **6.3**+**6.4**:**6.1**+**6.2.** While neither treatment yields any significant activation of PPARγ, both treatments induce significant activation of PPARα. Importantly, the activation rendered by the isomer mixture is significantly higher than that of compound **6** alone. This could provide an explanation for the greater decrease in 231 cell proliferation seen with the isomer mixture.

3.2.3 CONCLUSIONS

Taken together, these data suggest two things about the structure activity relationship of compound **6** and FABP5: that the endo conformation at carbons 2 and 3 is required for proper inhibition and the 2S, 3R regioisomer is preferred. Further

specification may be made clear with purification of the 9R/S stereoisomers. It was also demonstrated that the methyl-cyclohexane group is required for proper any inhibitory activity.

The activity of each isolated isomer mixture is unable to reproduce the same growth inhibitory or gene expression effects seen with commercial compound **6**, which indicates that the small amount of **6.1+6.2** seen with **6.3+6.4** in the NMR spectra of compound **6** may play a large part in the inhibitory activity of commercial compound **6**. When the synthesized endo isomers are mixed in the same 9:1 ratio as seen in compound **6,** they fail to reduce cell proliferation in the same manner (Figure 3.10). This indicates that the chirality of carbon 9 may play a large role in compound **6**'s inhibitory activity. Further experiments should isolate endo isomers with specific carbon 9 chirality in order to test this hypothesis.

Interestingly, the *2S,3R* endo isomer mixture **6.1+6.2** shows an improved decrease in PPARδ target genes compared to commercial compound **6**, but this improvement is not reflected in cell proliferation. The NMR spectrum of compound **6** clearly displays that the compound is composed mostly of the *2R,3S* isomer **6.3+6.4**, but there is a small amount of **6.1+6.2** present in an approximate 1:9 ratio. Together, these data suggest that a combination of **6.3+6.4** and **6.1+6.2** in different ratios may improve inhibitor performance.

When varying ratios of the two isomer mixtures are incubated with MDA-MB-231 cells, a 1:2 mixture of **6.3+6.4:6.1+6.2** significantly decreases 231 cell proliferation when compared to compound **6** or either isolated isomer. The expression of PPARδ and RAR target genes remains largely unchanged when compared to compound **6**, suggesting

that the 1:2 mixture may work to inhibit cell proliferation outside of the FABP5/PPARδ signaling pathway. This mixture induces significantly higher activation of PPARα when compared to compound **6**. PPARα is associated with anti-proliferative activity in certain tumor types and environments and plays a large role in fatty acid β-oxidation and lipid homeostasis, so a compound that targets this nuclear receptor could be a good candidate in treating other cancers.[13–16] Future experiments should verify this PPARα activity by measuring expression of its target genes. Because there is no previously reported interaction between PPARα and FABP5, further experiments should also look for cross-activation between the two.

3.3 Experimental Methods

3.3.1 General Experimental Methods

All cells were cultured in DMEM with L-glutamine and 4.5g/L glucose without sodium pyruvate supplemented with 10% FBS and 100IU penicillin/100mg/mL streptomycin in a humidified atmosphere at 37°C and 5% CO_2, unless otherwise stated. Absorbance, fluorescence, and luminescence readings were performed on a Molecular Devices SpectraMax i3x Multimode detection platform. All statistical analyses were calculated with GraphPad Prism software.

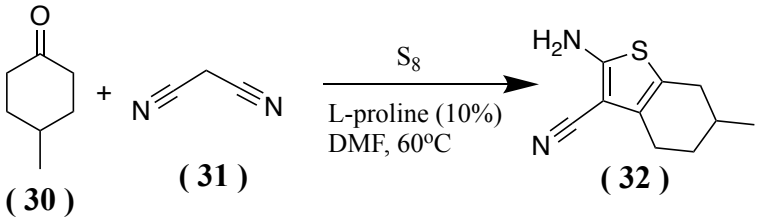
3.3.2 Reagents and Chemicals

MDA-MB-231 and Cos7 cells were purchased from ATCC. DMEM, penicillin-streptomycin 100x solution, and 2.5% trypsin solution were purchased from Corning. US-origin FBS was purchased from VWR. PrestoBlue Cell Viability reagent, TriZol, and B-PER were purchased from ThermoFisher Scientific. IPTG, ampicillin, and imidazole

were purchased from Sigma Aldrich. Ni Sepharose 6 FastFlow Beads were purchased from GE Life Science. The luciferase reporter assay system was purchased from Promega. iScript cDNA Synthesis kit was purchased from BioRad.

All synthetic protocols and characterizations described below were designed and performed by Yuzhi Shang, Yongchun Hou, and Emma Whiting.

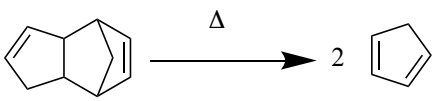
3.3.3 SYNTHESSIS OF AMINE INTERMEDIATE (32)



(30) **(31)** **(32)**

4-methylcyclohexanone **(30)** (1.75mL, 14.26 mmol), malonoitril **(31)** (1.04g, 14.69 mmol), cyclo-octasulfur (0.686g, 21.39mmol), L-proline (0.164 g, 1.43mmol) and DMF (15mL) were added to a 100mL round bottom flask heated to 60°C with stirring overnight. The reaction was quenched with water and extracted with ethyl acetate 5 times. Organic layers were combined, dried with sodium sulfate, filtered and dried under vacuum. The amine intermediate **(32)** was purified by CombiFlash using hexanes and ethyl acetate. 97.5%. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.31 (s, 2H), 3.52 (s, 2H), 3.48 (s, 2H), 2.80-2.75 (dd, 2H), 2.65-2.58 (m, 1H), 2.32-2.37 (dd, 1H), 1.92 (d, 2H), 1.79 (d, 1H), 1.61 (d, 1H), 1.46-1.38 (dddd, 1H), 1.08 (d, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 175.05, 137.01, 136.35, 135.19, 135.05, 112.92, 109.49,52.31, 46.32, 45.85, 32.86, 30.12, 29.45, 24.27, 21.34.

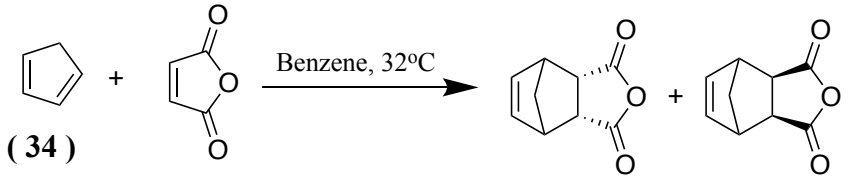
3.3.4 SYNTHESIS OF CYCLOPENTADIENE (34)



(33) **(34)**

Dicyclopentadiene (**33)** (10.10g, 76mmol) was added to a short-stem fractional distillation apparatus in a 0°C ice bath and heated to 190-200°C. Cyclopentadiene **(34)** distilled at 40°C as a clear, colorless liquid with a yield of 88.4%. ^{1}H-NMR (500MHz, $CDCl_3$): δ 6.58 (d, J=5.0 Hz, 2H), 6.47 (d, J=5.0 Hz, 2H), 2.99 (t, 2H); ^{13}C-NMR (126 MHz, $CDCl_3$): δ 133.19, 132.27, 41.64.

3.3.5 SYNTHESIS OF ENDO (36) AND EXO (37) ANHYDRIDE

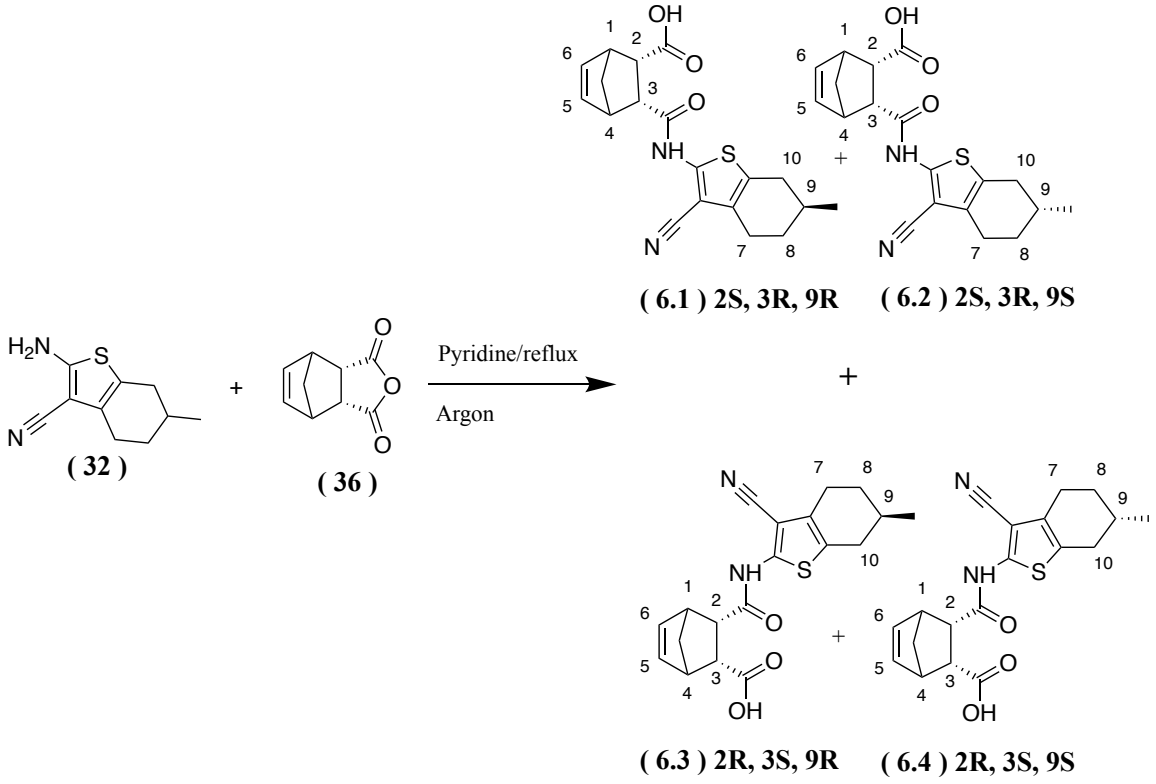


(34) **(35)** **(36)** **(37)**

Maleic anhydride **(35)** (1.56g, 16mmol, 1eq.) was dissolved in 3.5mL of benzene and heated to 32°C. Cyclopentadiene **(34)** (2.3mL, 27mmol, 1.7eq.) was added dropwise to the mixture at a rate of 5 drops/15 minutes for a total of two hours. The reaction mixture was then agitated at room temperature for 3 days to produce a mixture of the endo anhydride (**36)** and exo anhydride **(37)** as a white crystalline solid with a yield of 99.6%. Endo and exio anhydrides were purified by repeated flash column separation using 8.5:2:1 hexanes:acetone:ethyl acetate. Endo **(36)**: ^{1}H-NMR (500MHz, $CDCl_3$): δ 6.32 (d, *J*=2.1Hz, 2H), 3.58 (d, *J*=2.9Hz, 2H), 3.54-3.49 (m, 2H), 1.79 (d, *J*=9.0Hz, 1H), 1.57 (d, *J*=9.0Hz, 1H); ^{13}C-NMR (126 MHz, $CDCl_3$): δ 171.24, 135.54, 52.76, 47.08, 46.14. Exo

(**37**): ^{1}H-NMR (500MHz, $CDCl_3$): δ 6.33 (t, *J*=2.0Hz, 2H), 3.46 (t, *J*=2.0Hz, 2H), 3.00 (d, J=2.0Hz, 2H), 1.67 (dt, *J*=10.3, 1.9Hz, 1H), 1.45 (d, *J*=10.2Hz, 1H)

3.3.6 SYNTHESIS OF 6.1+6.2 AND 6.3+6.4



(32) **(36)**

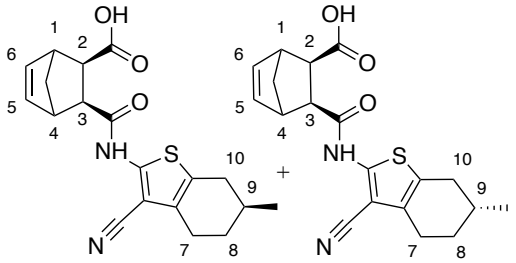
(6.1) 2S, 3R, 9R **(6.2) 2S, 3R, 9S**

(6.3) 2R, 3S, 9R **(6.4) 2R, 3S, 9S**

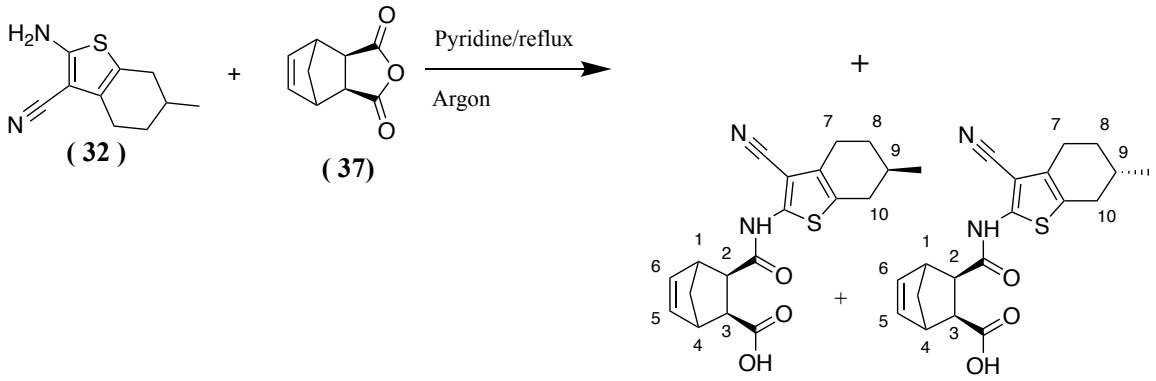
An equal molar ratio of endo-anhydride **(36)** and amine intermediate **(32)** were mixed in 10mL dry pyridine in a dry round bottom flask. The mixture was heated to reflux at 115^{o}C for 20hr. The product was dried under vacuum. The racemic mixtures of **6.1+6.2** and **6.3+6.4** were purified by CombiFlash chromatography in hexanes/ethyl acetate from 5-25%. **6.1+6.2**: ^{1}H-NMR (500MHz, MeOD): δ 6.27 (t, *J*=1.9 Hz, 2H), 3.58 (dd, *J*=2.9, 1.6 Hz, 2H), 3.44 (dq, *J*=3.6, 1.9 Hz, 2H), 2.86 (dd, *J*=16.6, 5.0 Hz, 1H), 2.75 (ddt, *J*=16.8, 4.8, 2.5 Hz, 1H), 2.01-1.93 (m, 2H), 1.75 (dt, *J*=8.9, 1.8 Hz, 1H), 1.68 (d, *J*=8.8 Hz, 1H), 1.48 (dtd, *J*=13.6, 11.0, 5.6 Hz, 1H), 1.11 (d, *J*=6.5 Hz, 3H); ^{13}C-NMR (126

MHz, MeOD): δ 176.93, 172.1, 149.0, 138.48, 138.44, 136.15, 135.54, 113.79, 109.95, 53.04, 48.94, 48.83, 48.66, 48.59, 48.49, 47.48, 46.79, 33.46, 31.18, 30.67, 25.04, 21.42. **6.3+6.4**: ^{1}H-NMR (500MHz, MeOD): δ 6.25 (ddd, *J*=5.6, 2.9 Hz, 2H), 3.59-3.52 (m, 2H), 3.46-3.40 (m, 2H), 3.14 (d, *J*=12.9Hz, 4H), 2.66 (ddd, *J*=22.2, 14.7, 9.7 Hz, 2H), 2.53 (s, 1H), 2.20 (dt, *J*=16.1, 5.6Hz, 2H), 2.01(s, 1H), 1.91 (s, 3H), 1.43-1.37 (m, 3H), 1.30 (1H), 1.07 (d, 3H); ^{13}C-NMR (126 MHz, MeOD): δ 176.54, 172.91, 149.13, 136.75, 136.32, 132.34, 129.27, 115.64, 94.49, 51.70, 50.47, 50.23, 49.97, 49.80, 48.05, 33.38, 32.13, 31.47, 31.45, 25.32, 25.29, 22.10, 22.09.

3.3.7 SYNTHESIS OF 6.5+6.6 AND 6.7+6.8



(6.5) 2S, 3R, 9R **(6.6) 2S, 3R, 9S**



(32) **(37)**

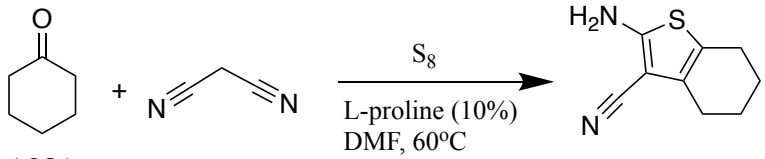
(6.7) 2R, 3S, 9R **(6.8) 2R, 3S, 9S**

6.5+6.6 and **6.7+6.8** were synthesized as racemic mixtures with exo-anhydride **(19)** and amine intermediate **(14)** via the same protocol used to synthesize **6.1+6.2** and **6.3+6.4**.

6.5+6.6: ^{1}H-NMR (500MHz, $CDCl_3$): δ 6.38 (t, *J*=1.9Hz, 2H), 3.37 (t, *J*=1.8Hz, 2H), 2.84 (d, *J*=1.3Hz, 2H), 2.74 (ddd, *J*=12.2, 9.3, 5.2Hz, 2H), 2.27 (ddt, *J*=17.1, 9.9, 2.0Hz, 1H), 1.92-1.82 (m, 2H), 1.58 (dt, *J*=10.2, 1.6 Hz, 1H), 1.53 (d, *J*=10.2 Hz, 1H), 1.38 (dtd, *J*=13.4, 11.1, 5.6 Hz, 1H), 1.03 (d, *J*=6.5Hz, 3H); ^{13}C-NMR (126 MHz, MeOD): 177.09, 175.55, 173.27, 148.11, 139.34, 139.32, 139.03, 138.76, 138.71, 135.49, 132.05, 129.25, 114.94, 113.77, 110.11, 94.68, 48.19, 47.00, 46.43, 45.56, 44.01, 33.50, 32.87, 31.59, 31.57, 31.18, 30.94, 30.92, 30.68, 25.04, 24.82, 21.60, 21.58, 21.44.

6.7+6.8: ^{1}H-NMR (500MHz, $CDCl_3$): δ 8.37 (s, 1H), 6.36-6.27 (m, 2H), 4.16-4.11 (m, 2H), 3.48 (d, *J*=15.3Hz, 1H), 3.23 (d, *J*=17.6Hz, 1H), 3.13 (d, *J*=11.3Hz, 1H), 3.00 (d, *J*=2.0Hz, 1H), 2.92 (td, *J*=13.8, 5.8Hz, 1H), 2.77 (d, *J*=14.7Hz, 1H), 2.72-2.65 (m, 2H), 2.56 (s, 1H), 1.90 (s, 1H), 1.77 (t, *J*=13.1 Hz, 1H), 1.26 (s, 1H), 1.24 (dd, *J*=20.4, 14.3 Hz, 1H), 1.05 (dd, *J*=19.1, 6.5Hz, 3H); ^{13}C-NMR (126 MHz, $CDCl_3$): δ 176.7, 172.9, 146.8, 135.9, 135.4, 128.9, 116.9, 67.8, 53.7, 49.8, 47.2, 45.7, 31.5, 28.4, 21.7, 20.4.

3.3.8 SYNTHESIS OF NON-METHYLATED AMINE INTERMEDIATE (39)

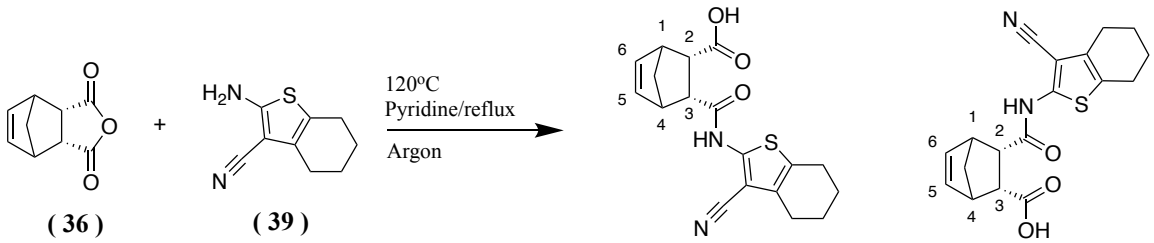


(38) **(31)** **(39)**

Non-methylated anhydride **(39)** was synthesized using cyclohexanone **(38)** in the same manner as the methylated amine intermediate **(32).**

^{1}H-NMR (500MHz, $CDCl_3$): δ 2.58 (m, 2H), 2.45 (m, 2H), 1.78 (t, *J*=7.0, 1.6 Hz, 4H); ^{13}C-NMR (126 MHz, $CDCl_3$): δ 148.5, 140.9, 134.3, 116.9, 82.8, 24.4, 23.4, 22.9.

3.3.9 SYNTHESIS OF 6.9 AND 6.10



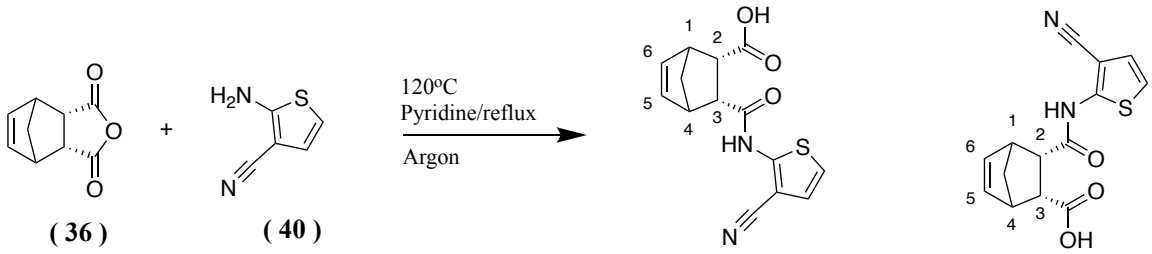
(36) **(39)** **(6.9) 2S, 3R** **(6.10) 2R, 3S**

The racemic mixture of non-methylated endo derivatives of compound 6 **(6.9+6.10)** was synthesized in the same manner as compounds **(6.1+6.2)** and **(6.3+6.4)** using the endo anhydride **(36)** and non-methylated amine intermediate **(39)**.

6.9: ^{1}H-NMR (500MHz, MeOD): δ 6.27 (t, *J*=1.9 Hz, 2H), 3.58 (dd, *J*=2.9, 1.6 Hz, 2H), 3.44 (dq, *J*=3.6, 1.9 Hz, 2H), 2.86 (dd, *J*=16.6, 5.0 Hz, 1H), 2.58 (m, 2H), 2.45 (m, 2H), 2.01-1.93 (m, 2H), 1.78 (t, *J*=7.0, 1.6 Hz, 4H); ^{13}C-NMR (126 MHz, MeOD): δ 176.5, 172.8, 145.8, 135.9, 135.3, 128.9, 116.9, 67.8, 53.7, 49.8, 47.2, 45.7, 24.4, 23.4, 22.9.

6.10: ^{1}H-NMR (500MHz, $CDCl_3$): δ 6.38 (t, *J*=1.9Hz, 2H), 3.37 (t, *J*=1.8Hz, 2H), 2.84 (d, *J*=1.3Hz, 2H), 2.74 (ddd, *J*=12.2, 9.3, 5.2Hz, 2H), 2.58 (m, 2H), 2.45 (m, 2H), 2.01-1.93 (m, 2H), 1.78 (t, *J*=7.0, 1.6 Hz, 4H); ^{13}C-NMR (126 MHz, MeOD): δ 176.6, 172.7, 145.8, 135.7, 135.3, 128.9, 116.8, 67.7, 53.7, 49.8, 47.2, 45.5, 24.4, 23.4, 22.8.

3.3.10 SYNTHESIS OF 6.11 AND 6.12



(36) **(40)** **(6.11) 2S, 3R** **(6.12) 2R, 3S**

Non-cyclo endo derivatives of compound 6 **(6.11)** and **(6.12)** were synthesized in the same manner as compounds **(6.10)** and **(6.9)** using 2-amino-3-thiophenecarbonitrile **(40)** and endo anhydride **(36).** The crude mixture of **6.11** and **6.12** was purified using CombiFlash chromatography in hexanes/ethyl acetate from 0-20%.

6.11: ^{1}H-NMR (500MHz, MeOD): δ 7.65 (d, *J*=5.7 Hz ,1H), 7.31 (d, *J*=5.7 Hz,1H), 6.28 (t, *J*=2.0 Hz, 2H), 3.60 (dd, *J*=3.0, 1.6 Hz, 2H), 3.45 (dq, *J*=3.5, 1.8 Hz, 2H), 1.77 (dt, *J*=8.8, 1.8 Hz, 1H), 1.69 (d, *J*=8.8 Hz,1H); ^{13}C-NMR (126 MHz, MeOD): δ 176.5, 172.9, 149.1, 127.9, 117.3, 115.3, 95.7, 53.7, 49.8, 47.2.

6.12: ^{1}H-NMR (500MHz, MeOD): δ 7.02-6.93 (m, 2H), 6.28(dd, *J*=5.6, 2.9 Hz, 2H), 3.60 (dd, *J*=10.1, 3.3 Hz, 1H), 3.46 (dd, *J*=10.2, 3.4 Hz, 1H), 3.34 (s, 1H), 3.16 (d, *J*=8.4 Hz,2H), 1.50-1.41 (m, 2H), 1.30 (d, *J*=11.5 Hz, 1H); ^{13}C-NMR (126 MHz, MeOD): δ 176.5, 172.9, 149.2, 127.7, 117.3, 115.4, 95.8, 53.7, 49.9, 47.2.

3.3.11 TRANSACTIVATION REPORTER ASSAY

Cos7 cells were cultured in charcoal treated media and co-transfected with vectors encoding a luciferase reporter driven by three copies of peroxisome proliferator response element (PPRE), an expression vector for PPARα, δ or γ, and an expression vector β-galactosidase as a transfection control. Transfected cells were cultured in serum-free media for 4 hrs. and then treated with serum-free media containing 1μM of the appropriate PPAR agonist or 20μM of the compound of interest for 18hrs. Following incubation, cells were lysed with 1xReporter Lysis Buffer. β-galactosidase activity was measured by incubated 30μL cell lysate with 100xMg solution (0.1M $MgCl_2$, 4.5M β-mercaptoethanol), 0.1M sodium phosphate (pH 7.5), and 1XONPG (4mg/mL o-nitrophenyl-β-D-galactopyranoside in sodium phosphate buffer) until a strong yellow color developed. Absorbance was read at 420nM. Luciferase activity of the lysates was measured using the luciferase reporter assay system and normalized to β-galactosidase activity. Values are calculated as a fold change relative to untreated cells.

3.3.12 PROLIFERATION ASSAYS

Cells were seeded at 5,000 cells/well in a 96-well plate and allowed to adhere overnight. Media containing the compound of interest was applied to the cells and changed every other day for 4 days. On the fourth day, PrestoBlue Cell Viability reagent was added to the cells and incubated for 60min. Fluorescence was read at λ_{ex}=560nM/λ_{em}=590nM. Fold proliferation is calculated relative to the highest fluorescence intensity among untreated cells.

3.3.13 qRT-PCR

MDA-MB-231 cells at 80-90% confluency were incubated with compound for 6hrs at 37^{o}C and 5% CO_2. Following incubation, total RNA was extracted using TRIZol reagent. RNA purity was evaluated via UV-Vis NanoDrop spectrophotometry. cDNA was synthesized from RNA using a High Capacity RNA-to-cDNA kit or an iScript cDNA synthesis kit and used in subsequent reactions at a 1:5 dilution. qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System with TaqMan assay probes and TaqMan Fast Advanced Master Mix. Fold change is calculated relative to expression levels of transcription control and the target gene in untreated 231 cells.

3.4 Selected ^{1}H-NMR Spectra of Synthesized Compound 6 Isomers

See Appendix.

3.5 Acknowledgments

Grateful acknowledgment to Yuzhi Shang, Yongchun Hou, and Emma Whiting for the design, preparation and characterization of compound 6 derivatives. Special thanks to Heather Folkwein-Kennehan for the growth and characterization of commercial compound **6**.

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CHAPTER 4. THESIS SUMMARY AND FUTURE DIRECTIONS

4.1 THESIS SUMMARY

All-trans retinoic acid (atRA) is the biologically-active metabolite of vitamin A responsible for initiating the expression of specific target genes. When retinoic acid receptor (RAR) is activated by atRA, it initiates the transcription of genes associated with cell cycle regulation and apoptosis, among others. In some instances, it is possible to utilize this signaling pathway to inhibit tumor progression. However, in certain cancers, atRA induces cancer cell growth and tumor progression.[1,2] This paradoxical activity of atRA was found to be due to the activation of two different signaling pathways: the cellular retinoic acid binding protein 2 (CRABP2)/retinoic acid receptor (RAR) pathway and the fatty acid binding protein 5 (FABP5)/peroxisome proliferator activated receptor δ (PPARδ) pathway.[3]

In canonical atRA signaling, atRA is bound by CRABP2 in the cytoplasm and shuttled into the nucleus where it activates the nuclear receptor RAR. When RAR is activated, it initiates the transcription of genes associated with tumor death. However, if FABP5 exists in significant excess of CRABP2, it will bind atRA and shuttle it to an alternate nuclear receptor, PPARδ.[3] When PPARδ is activated, it initiates the transcription of genes that promote cell proliferation and tumor growth. The central hypothesis of this dissertation asserts that in the case of FABP5 excess, we may be able to target FABP5 with a small-molecule inhibitor in such a way that prevents the activation of PPARδ and thereby eliminates atRA-induced pro-proliferative effects.

In order for any compound to be a good candidate FABP5 inhibitor, it must meet two criteria: 1) it must express high binding affinity toward FABP5, and 2) it must not

independently activate PPARδ. Effective inhibition of FABP5 is expected to disrupt the pro-proliferative PPARδ activity and re-direct atRA toward RAR, thereby activating anti-carcinogenic activities. After screening a variety of candidate inhibitors, both naturally occurring and synthesized, only compound **5**, or AM404, a metabolite of acetaminophen, and compound **6**, a multi-ringed amine compound, were able to reduce proliferation and PPARδ-activation in FABP5-rich MDA-MB-231 cells. While both compounds show promise as FABP5 inhibitors, compound **6** possesses more possible structural variations and would be a novel drug candidate should it prove effective against atRA-resistant cancers on a wider scale.

When exploring the structure-activity relationship of compound **6** and FABP5, it was found that the commercially available compound is *endo* at carbons 2 and 3 and is likely composed of a racemic mixture of the *2R,3S* isomers **6.3+6.4**. When each possible isomer mixture of compound **6** is tested with FABP5-rich MDA-MB-231 cells, both *endo* isomer mixtures and the *exo 2S,3R* isomer mixture were able to induce a reduction in cell proliferation. However, the **6.3+6.4** mixture is unable to induce a reduction in proliferation or significant change in PPARδ and RAR target gene expression as effectively as the commercial compound. The *2S,3R endo* mixture **6.1+6.2** actually inhibits proliferation most effectively amongst the possible isomer mixtures and shows a more significant reduction in the expression of PPARδ target genes when compared to the commercial compound. When endo isomers missing either the methyl group, **6.9** and **6.10,** or the methylcyclohexane group, **6.11** and **6.12**, are incubated with MDA-MB-231 cells, anti-proliferative activity is eliminated.

From this data, it is likely that an *endo* isomer is the most effective form of compound **6**, but the role of the stereochemistry of carbons 2 and 3 remains unclear. When examining the NMR-spectra of commercial compound **6**, there is actually a small amount of the **6.1+6.2** mixture present in an approximate 1:9 ratio with the majority **6.3+6.4** mixture, indicating that a mixture of *endo* isomers may be required to reproduce the inhibitory activity of commercial compound **6.** However, when different mixtures of **6.3+6.4**:**6.1+6.2** are incubated with 231 cells, the 9:1 mixture still fails to reproduce the reduction in proliferation seen with commercial compound **6**. Together, this data indicates that the chirality of carbon 9 on compound **6** likely plays a large role in the inhibitory activity of the compound.

While the mixtures of **6.3+6.4**:**6.1+6.2** were unable to replicate the activity of compound **6**, a 1:2 mixture of **6.3+6.4**:**6.1+6.2** dramatically improves anti-proliferative activity in MDA-MB-231 cells when compared to compound **6**. There is no significant change in PPARδ or RAR target gene expression after incubation with the 1:2 mixture, however. This indicates that the mixture may induce cell death outside of the FABP5/PPARδ signaling pathway. The 1:2 mixture induces a significant increase in PPARα activation when compared to the commercial compound, suggesting that PPARα activity may contribute to the anti-proliferative effects seen after incubation with the mixture.

4.2 FUTURE DIRECTIONS

In regards to understanding the structure-activity relationship of FABP5 and compound **6**, a key step in moving forward will be to obtain a crystal structure of the

commercial compound bound to the protein. This will elucidate the key interactions of the compound with the protein, which will provide further information that may be used to optimize inhibitory activity. Additionally, a binding assay and crystal structures with purified human FABP5, compound **6** and its synthesized isomers could provide further clarification on which isomers or isomer mixtures of compound **6** are responsible for its anti-proliferative properties.

An interesting development in the course of this research has been the activation of PPARα by compound **6** and the 1:2 mixture of **6.3+6.4:6.1+6.2**. While the activation rendered by commercial compound **6** is not statistically significant like that of the 1:2 mixture, PPARα activity is still elevated. This activation is particularly interesting given the biological role of the nuclear receptor. PPARα is mainly expressed in tissues that have high fatty acid β-oxidation rates, such as heart and skeletal muscle, endothelial cells, and brown adipose. It plays a major role in regulating lipid and glucose metabolism and can modulate inflammation pathways.[4,5] PPARα is a particularly interesting target in the context of cancer treatment given the metabolic switch in cancer cells to increase glucose and lipid metabolism.[6,7] The fatty environment of breast cancers mean that fatty acid uptake and homeostasis is especially dysregulated.[8,9] If compound **6** or any of its derivatives are able to effectively target PPARα-regulated aspect of fatty acid homeostasis in cancer, it could prove to be a new therapeutic target. Furthermore, like the FABP5/PPARδ pathway, PPARα activation can be regulated by cooperation with FABP1.[10,11] If compound **6** or any combination of its isomers are able to regulate PPARα via FABP5, it would be a novel intracellular lipid-binding protein/nuclear receptor interaction. Further studies of compound **6** should investigate this possibility.

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APPENDIX. SELECTED NMR SPECTRA OF COMPOUND 6 ISOMERS AND DERIVATIVES

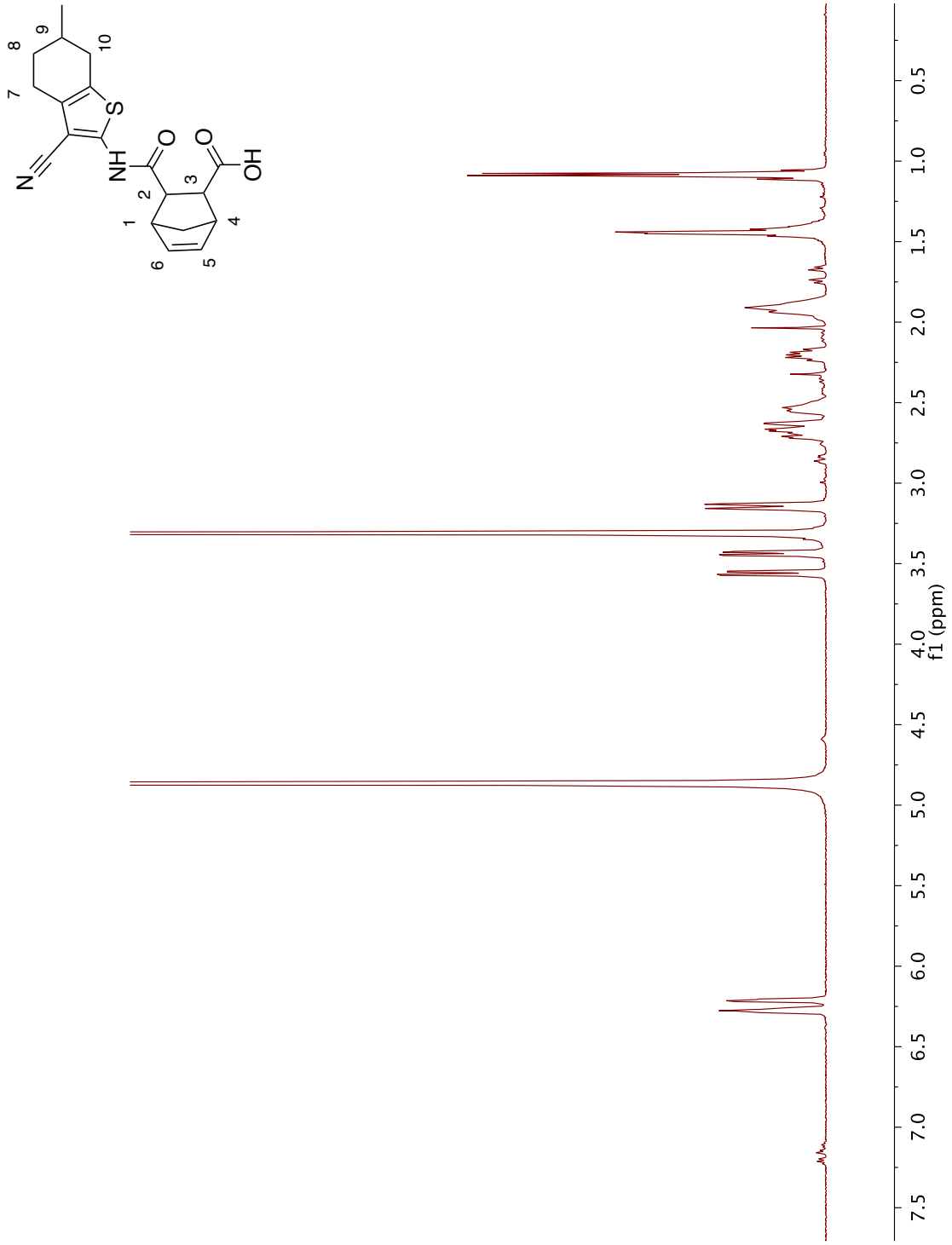

Figure A.1 ^{1}H-NMR of Compound **6** in MeOD.

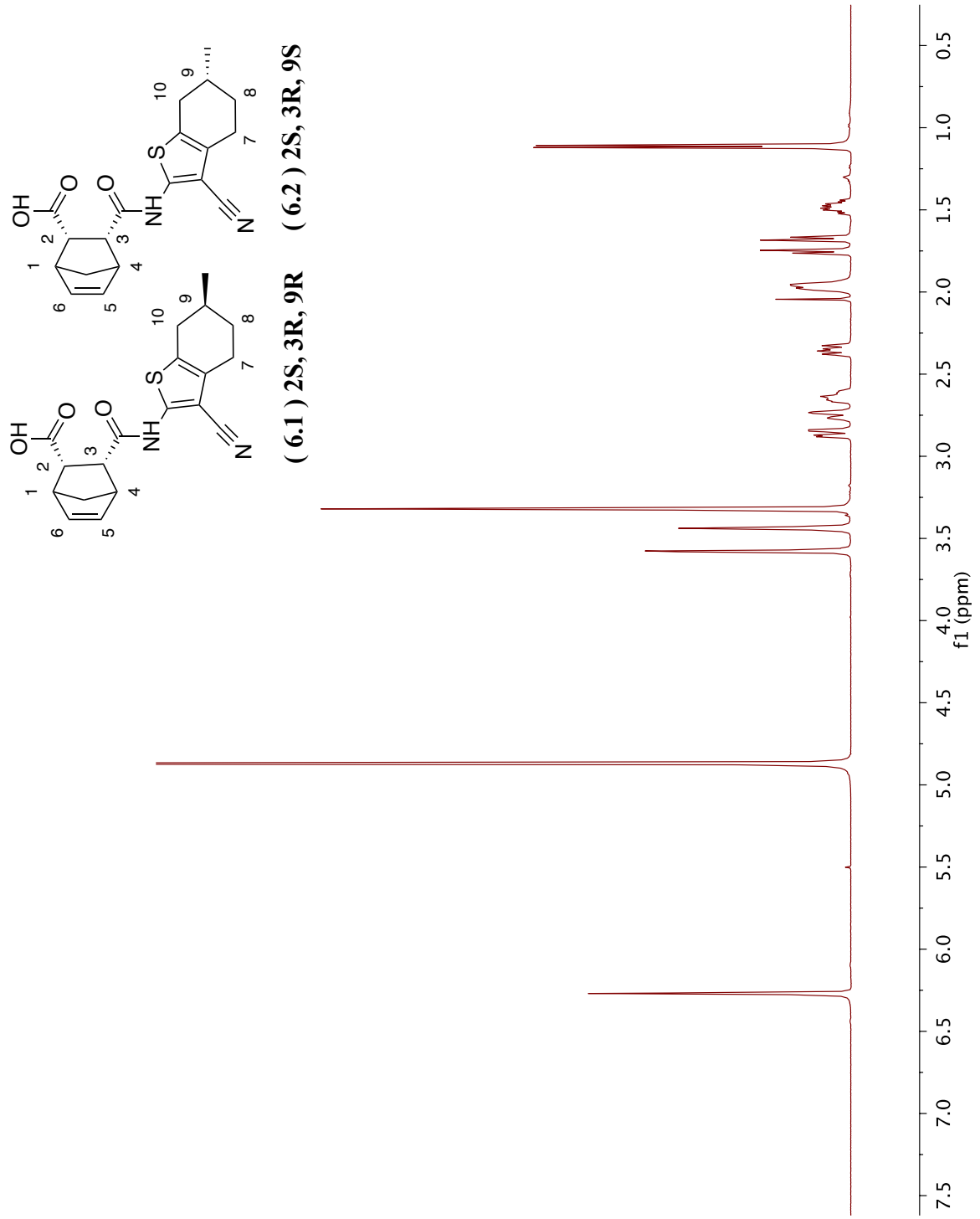

Figure A.2. ^{1}H-NMR of **6.1+6.2** in MeOD.

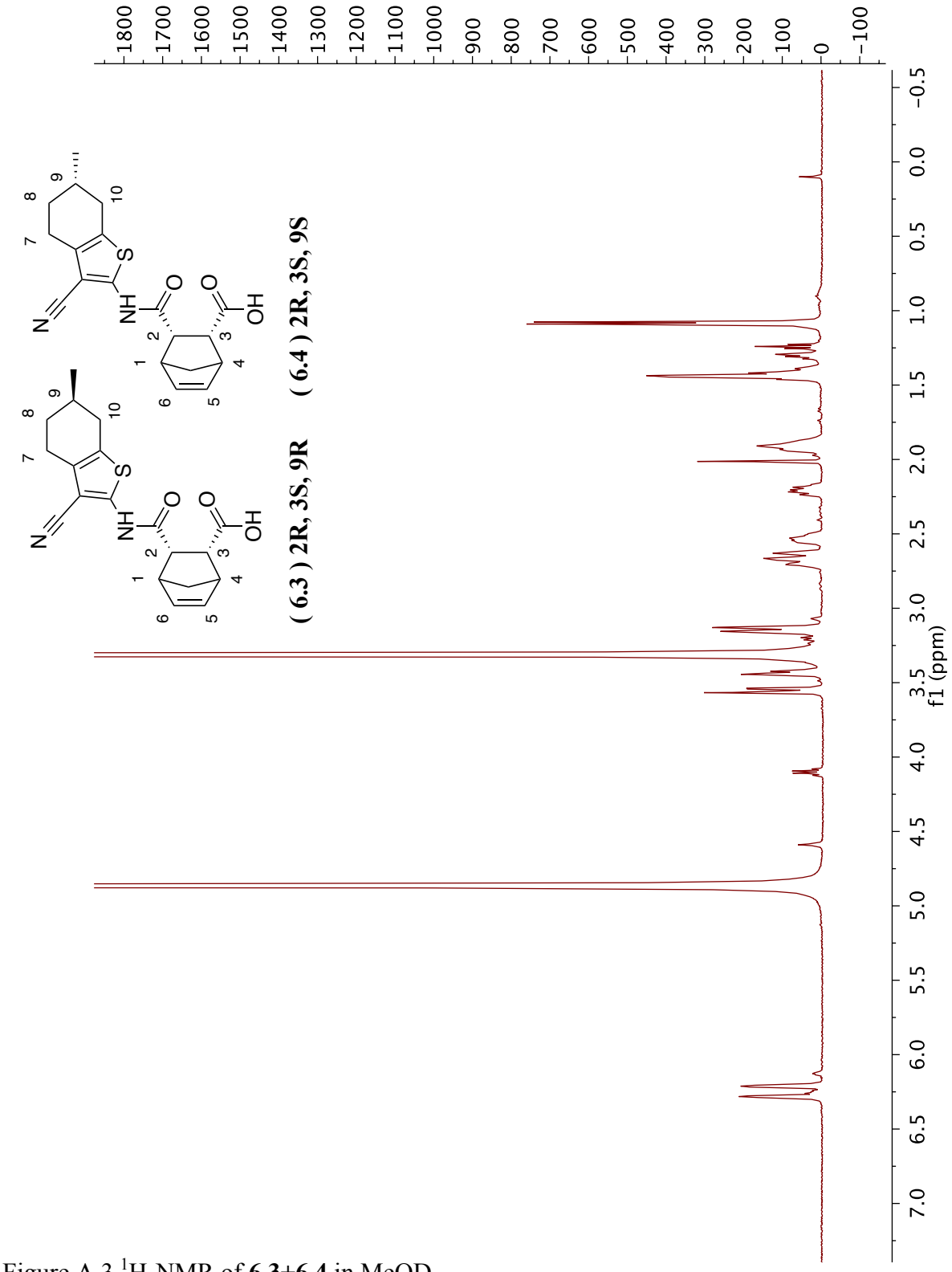

Figure A.3 ^{1}H-NMR of **6.3**+**6.4** in MeOD**.**

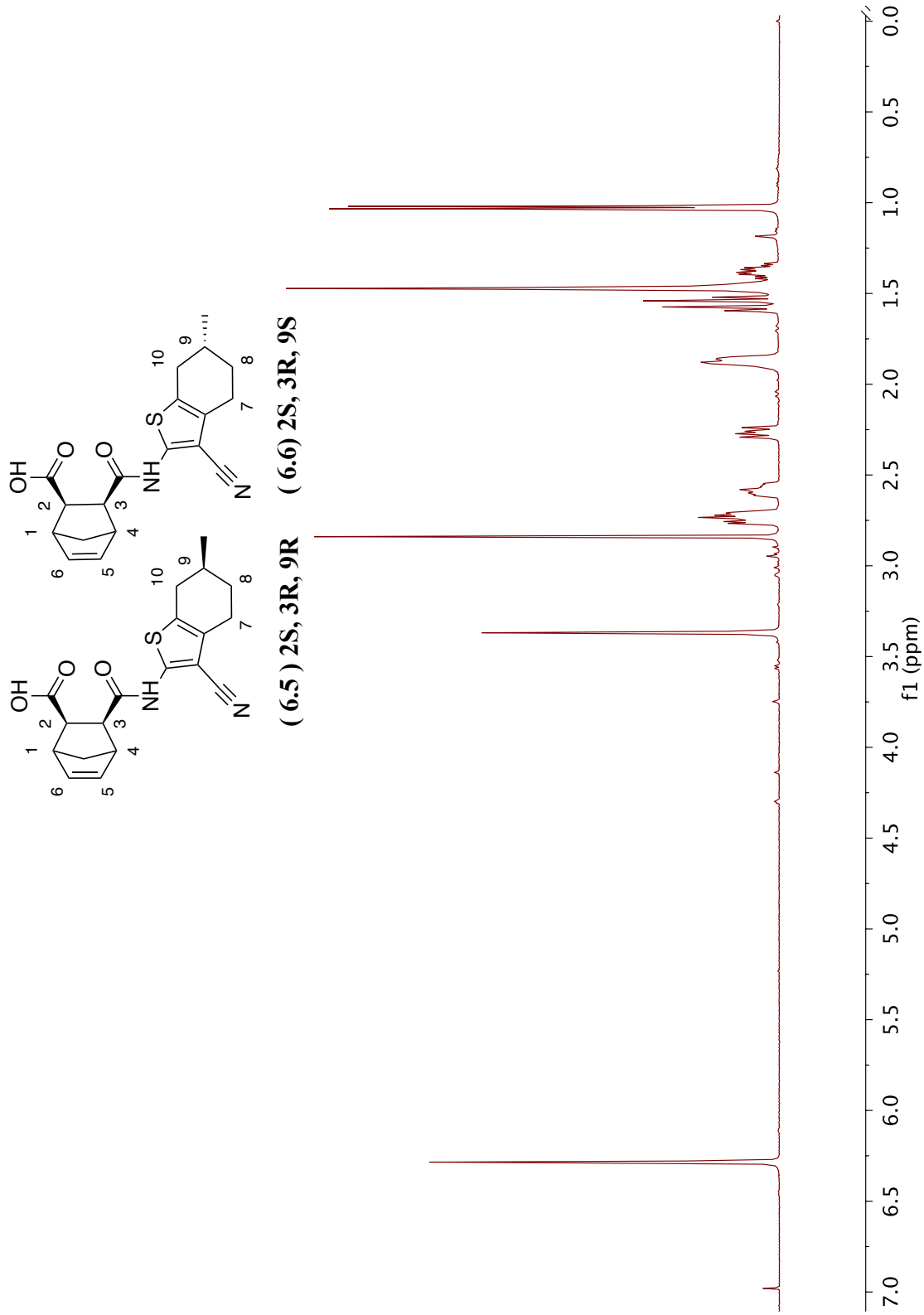

Figure A.4 ^{1}H-NMR of **6.5+6.6** in MeOD.

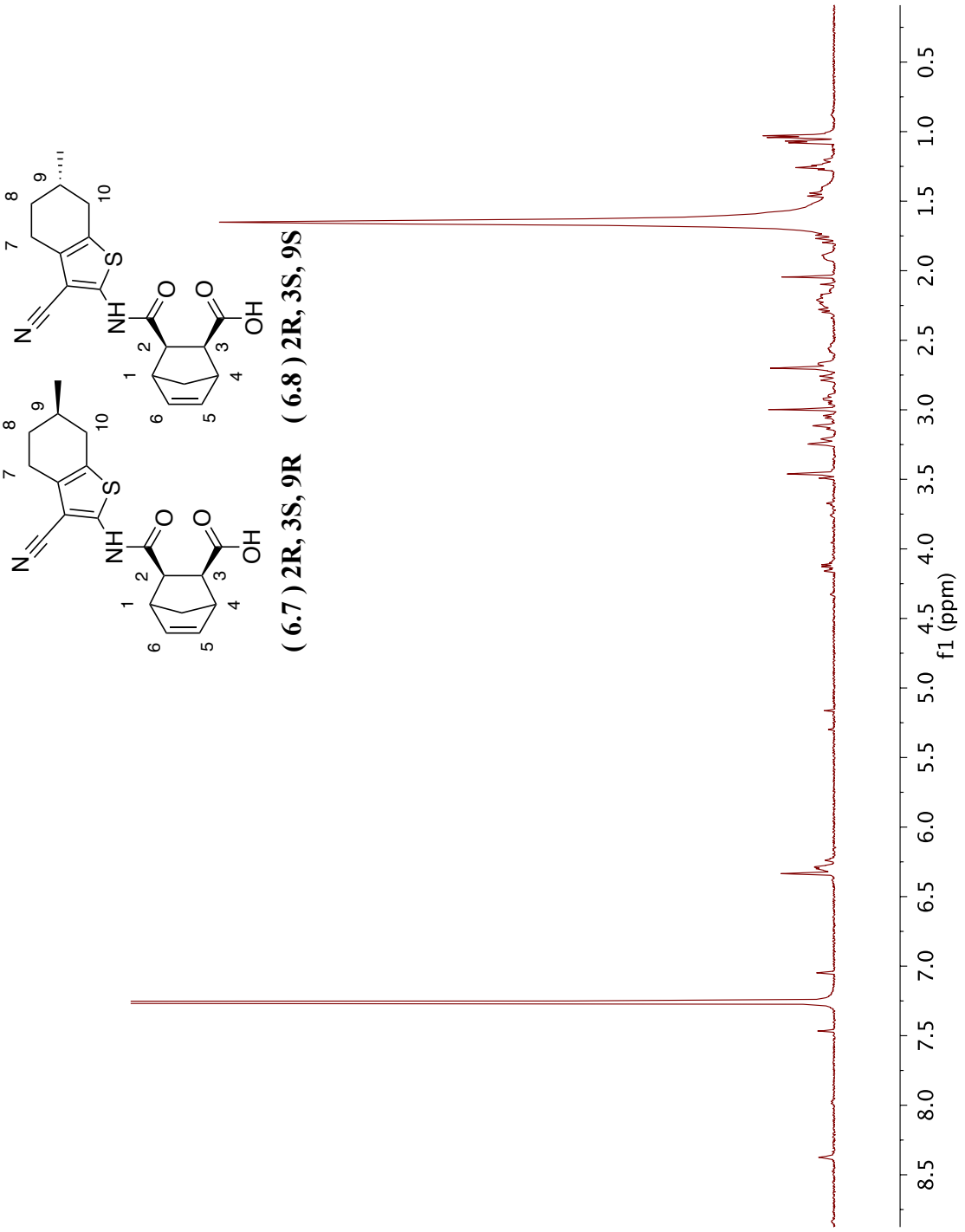

Figure A.4 ^{1}H-NMR of **6.7+6.8** in MeOD.

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