OPTIMIZING THYROID HORMONE RECEPTOR PROBES FOR USE IN FLUORESCENT LABELLING

by

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ABSTRACT

There has been renewed interest in the fluorescent thyroid hormone receptor probe, JZ01, previously developed in the Koh lab. This probe selectively binds to the thyroid hormone receptor, TR, and is useful in labeling and isolating cells involved in various cancers. With cells labelled by a fluorescent probe, researchers can use fluorescence-activated cell separation (FACS) to isolate their cells of interest. The first goal of this project is to resynthesize JZ01 for use in cell isolation.

A second goal of this project is to create a more selective labelling technique. It has become apparent that the selectivity of JZ01 between TRα and TRβ subtypes could use improvement. Collaborators began facing issues when using the newly synthesized JZ01 in their studies as they intended to differentiate cells overexpressing TRβ alone. Ideally, this fluorescent label would selectively bind the thyroid receptor β over its α subtype.

To block the fluorescent labelling of the thyroid receptor α, CO31 was synthesized. This triiodothyronine (T3) analog was previously created by Cory Ocasio, another Koh Group graduate. His research found that CO31 formed selective interactions with TRα that were ten to twenty-times greater than interactions with the TRβ subtype. Using these molecules in conjunction, CO31 would theoretically form selective interactions with TRα that would allow JZ01 to more selectively label TRβ in cells. With a more selective probe, collaborators would be able to more efficiently use FACS sorting in their studies.

The third goal of this project is to create improvements in the thyroid hormone probe itself. JZ01 is a fluorescein analog which presents several issues when working with cells. First, fluorescein is susceptible to photobleaching meaning it is moderately
unstable and prone to breakdown\textsuperscript{[2,4]}. Additionally, this analog can prove to be toxic to cells and its hydrophobic qualities hinders its labelling of cells\textsuperscript{[4]}. Therefore, to create an optimal tool for FACS sorting, it is necessary to synthesize analogs that are better taken up by cells, are less toxic at concentrations use to label, and are more structurally stable.

One such analog would include a BODIPY label. Using this analog would theoretically improve labeling by reducing the cellular toxicity associated with the fluorescein. Additionally, BODIPY is a more stable fluorescent analog meaning that the final label will show an increase in stability\textsuperscript{[2,6]}. Finally, BODIPY is known for having a high fluorescent quantum yield even in water meaning that this probe will be able to near 100\% efficiency, even in water\textsuperscript{[6]}. SF01 was created by making small alterations in the synthesis of JZ01 to more efficiently couple BODIPY in a final step. The same scaffold structure is used meaning that a relatively similar utility should be expected when using this BODIPY coupled probe.
Chapter 1
INTRODUCTION

1.1 Background

1.1.1 Relevance of thyroid hormone

Thyroid hormone is essential in the development of the adolescent brain and functions to remyelinate the adult brain. Because of its role in the healthy development and regulation of the body, cases of hypothyroidism can lead to poor brain development, fatigue, depression, and weight-gain. Conversely, hyperthyroidism can cause sensitivity to heat, heart problems, and anxiety [16,18]. Throughout history, impaired brain development associated with hypothyroidism has been a major issue and was addressed by the commercial iodization of table salt in the United States in 1924. This fortification resulted in an average increase of 3.5 IQ points nationwide in the United States [11].

1.1.2 Metabolism

The two most common thyroid hormones are tetraiodothyronine (T4) and triiodothyronine (T3). While T4 is found at concentrations 20 times higher than T3 in the blood, T4 is converted to the more potent T3 in the cell. This conversion is catalyzed by a deiodinase which removes an iodine from T4 to form T3 [9,16]. Triiodothyronine then functions as the ligand for both thyroid hormone receptors TRα and TRβ. These receptor subtypes are very similar in structure and their internal
ligand binding pockets only differ by one amino acid residue which makes creating selective ligands difficult \[9\].

![T4 and T3 structures](image)

Figure 1: Structures of Thyroid Hormones T4 and T3

### 1.2 Thyroid hormone receptors and cancer

#### 1.2.1 Thyroid hormone receptors

Thyroid hormone receptors TR\(\alpha\) and TR\(\beta\) are very similar in that they only differ by one residue in their ligand binding pockets \[9\]. They function to bind T3, along with cofactor proteins such as coactivators and corepressors, to modulate cell proliferation, differentiation, and cell death \[16\]. The receptors localize in the nucleus of the cell and are capable of regulating gene transcription by directly binding DNA when bound with ligand. Prior studies with JZ01 support the notion that these hormone receptors localize in the nucleus \[18\].
Despite their structural similarity, the occurrence of the two subtypes of hormone receptor varies between different tissues. Whereas TRα is predominantly found in the brain, heart, and bone, TRβ is the predominant subtype in the liver and other peripheral tissues. This difference in expression is of interest as the basis for differential labelling of tissue types would be possible with selective thyroid hormone receptor probes. The effects of hypothyroidism and hyperthyroidism could be more thoroughly addressed by observing the expression of TRα and TRβ subtypes in abnormal tissues.

1.2.2 Expression of TRα and TRβ

Thyroid hormone receptors are expressed as TRα and TRβ subtypes encoded by genes on chromosome 17 and 3, respectively. In particular, TRβ is susceptible to
mutation due to its position on the chromosome (3p21-25) as mutations in this gene can lead to a protein that has little to no function \cite{16}. This adverse mutation can lead to several diseases such as resistance to thyroid hormone syndrome along with several types of cancer \cite{9,16}.

While it is difficult to generalize the expression of thyroid hormone across all cancers, it is known that TRβ has a tumor-suppressing role in cells. Generally, downregulation of TRβ or a mutation in the gene encoding TRβ would promote hypothyroidism that, in turn, increases the metastases of tumor cells \cite{16}. Specifically, mutations in TRβ have been found in thyroid, lung, liver, and breast cancers as well as clear cell renal cell carcinomas (ccRCC) \cite{5,16}. Kamiya et al. report that 40.9\% of their sequenced ccRCC cells had at least one mutation in the TRβ gene specifically. Additionally, researchers found that these mutants were less capable of binding thyroid hormone and showed diminished TR activity \cite{5}.

It is essential that the relationship between thyroid hormone receptors and cancer be more thoroughly investigated as a common treatment in ccRCC causes hypothyroidism. Generally, tyrosine kinase inhibitors such as sorafenib, sunitinib, pazopanib, and axitinib cause thyroid disfunction in a mechanism that is thought to alter the metabolism of T4 to T3 \cite{16}. As hypothyroidism is thought to accelerate metastases of tumor cells, this current treatment may be counterproductive.

1.3 Fluorescent thyroid hormone receptor probes

1.3.1 JZ01

JZ01 was originally synthesized and characterized by Jianfei Zheng, a graduate student in the Koh Group in 2008. This probe was created to directly visualize TRβ in
cells and to serve as a nonradiometric probe for visualization in vitro. After testing several scaffolds that mimicked TR binding compounds, Zheng found that scaffolds modelled after the ligand NH-2 worked much better than their GC-1 and T3 counterparts\cite{10,18}. The fluorescent probe JZ01 was created to bind TRβ with a $K_d$ of $21.4 \pm 5.6$ nM\cite{18}. The IC50 of JZ01, however, was not able to be measured directly because it tended to form aggregations at high concentrations. To determine an approximate IC50, JZ07 was created using the same scaffold with a propargyl group replacing the fluorescein label. This assay showed that the JZ01 label had a specificity of 12.8 times for TRβ over TRα ($IC_{50\alpha}/IC_{50\beta}=12.8$). Ultimately, JZ01 was found to be the first subtype selective fluorescent thyroid hormone receptor probe allowing for the preferential staining of tissues, cells, and, solutions containing TRβ\cite{18}.

![NH-2 scaffold](image)

Figure 3: NH-2 scaffold as described by Nguyen

The first step in this project was to resynthesize JZ01 for collaborators using this probe to label cells. These groups were attempting to separate cancerous precursor cells that overexpress TRβ using fluorescence activated cell sorting (FACS).
As their cells of interest overexpress TRβ, it is important to have selectivity between TRα and TRβ subtypes. Initial tests showed that JZ01 also labelled cells expressing TRα which raised concerns about its selectivity. Despite a reported 13-fold selectivity for TRβ, it seems that the probe can be improved by eliminating any labelling of TRα.

1.3.2 SF01

As Zheng found that JZ01 was effective in labelling TR, the NH-2 scaffold can also be modified to accommodate other fluorophores that improve JZ01’s utility. While JZ01 uses a fluorescein fluorophore, SF01 uses a BODIPY fluorophore. BODIPY is known to be less toxic and more stable which is clearly desirable in a cellular label [4,6]. A BODIPY probe may prove to be less hydrophobic than JZ01 and less prone to forming undesirable aggregations. Additionally, BODIPY is known to have high quantum yields in water as well as sharp excitation and emission peaks that contribute to its brightness and effectiveness as a probe [6].

1.4 Fluorescence activated cell sorting

Fluorescence-activated cell sorting is a type of flow cytometry that uses a laser to excite fluorescently labelled cells. A heterogenous mixture of cells is labelled with a fluorescent molecule specific to the cell type of interest. The mixture is then loaded into the instrument which passes cells along in a stream of liquid where they are analyzed using a laser specific to the wavelength of the fluorescent label. Cells containing the label of interest can be separated into distinct collection vessels [15].

1.5 CO31

The strategy used in this project to create a more selective probe takes advantage of a second molecule, CO31, that forms selective interactions with TRα.
CO31 was synthesized by Cory Ocasio in 2008 as part of the Scanlan Group at University of California San Francisco. In his research, Ocasio was synthesizing TRα-specific thyromimetics. One compound, CO31, was found to have a 10-20 fold preference for TRα over TRβ in U2OS and HeLa cells (EC50 β/EC50 α)\textsuperscript{[12,13]}. In Zheng’s studies, the addition of a high concentration of T3 was utilized to show that JZ01 was indeed binding TR\textsuperscript{[18]}. Similarly, if CO31 was added in an appropriate quantity, it should be able to competitively block labelling of TRα over TRβ. In turn, the probe’s 13-fold TRβ selectivity should be increased significantly by a preferential blocking of TRα.
Chapter 2
METHODS AND MATERIALS

2.1 Synthesis of JZ01

2.1.1 JZ01 synthesis scheme reported by Zheng

(a) (1) n-BuLi, THF/Hexane, (2) DMF; (b) TMSCLiN₂, THF; (c) (PPh₃)₂PdCl₂, Cul, Piperidine; (d) TBAF, THF; (e) Cs₂CO₃, tert-butyl bromoacetate, DMF/THF; (f) LiOH, H₂O/THF; (g) pentafluorophenyl trifluoroacetate, TEA, THF, 0 °C-RT; (h) 5-(aminomethyl)fluorescein hydrochloride, TEA, THF/DMF; (i) 6 N HCl(aq)/THF, 40°C.

Figure 4: Generalized synthesis scheme of JZ01 as described by Zheng [18]

2.1.2 Description of JZ01 synthesis

Referring to the method previously used by Zheng et al., a 10mg sample of purified JZ01 was created. The method utilized in this project used 2-isopropylphenol, 4-hydroxy-2,6-dimethylbenzaldehyde, and 6-phenylhexanoic acid as
starting materials to create the NH-2 scaffold. The full synthesis scheme employed in this project is shown as follows in Figure 5. In total, this synthesis involved sixteen reactions and $^1$H NMR and $^{13}$C NMR spectra were taken of all intermediates to confirm their identity. After the final deprotection to yield JZ01, the product was purified using HPLC and characterized again using $^1$H NMR and $^{13}$C NMR. This fluorescein analog is light-sensitive and was stored in darkness at -20°C.
Figure 5A: Synthesis of JZ01
Figure 5B: Synthesis of JZ01 (cont.)
2.2 Synthesis of CO31

2.2.1 Description of CO31 synthesis

In Ocasio’s original description of his synthesis of CO31, a generalized scheme of his thyromimetic compounds shows a seven-step synthesis involving the protection and deprotection of the alcohol and amine groups of triiodothyronine (T3).

![Chemical Reaction Diagram]

Beginning with T3, this scheme was followed without much success. Starting material and intermediates had solubility issues even at a small scale. Additionally,
with protection and deprotection of intermediates, yields were very low and it was impractical to arrive at a final product. To achieve better yields and synthesize CO31 in a more reasonable matter, the scheme was revised with the help of Raghu Neelarapu to shorten the synthesis and remove unnecessary protection of intermediates. While unprotected materials risked side reactions, TLC, column chromatography, \(^1\)H NMR, and \(^{13}\)C NMR were used at every step of this synthesis to ensure that a pure product was created.

Figure 7: Synthesis of CO31 used in this project
2.2.2 Methyl 2-amino-3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)propanoate

To a solution of triiodothyronine (300mg, 0.376mmol) in MeOH (20mL), 5 drops of HCl were added and the solution was heated at reflux (70°C) for 36 hours until TLC showed completion. The reaction mixture was neutralized with NaHCO₃ and excess MeOH was evaporated under reduced pressure. The remaining mixture was washed with 0.1N HCl and extracted using ethyl acetate. Organic fractions were dried over MgSO₄ and evaporated under reduced pressure to yield a fine white powder, 1 (250mg, 81.6%).

2.2.3 2-amino-3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)propenamide

Product 1 (100mg, 0.150mmol) was dissolved in 7N NH₃/MeOH (5mL) and the solution stirred at room temperature for 48 hours until observed to be complete by TLC. The MeOH was evaporated off under reduced pressure. The crude product was purified using column chromatography and an amine stationary phase (0-20% methanol in dichloromethane). The pure fractions were evaporated under reduced pressure to yield 2 (100mg, 100%).
In a Pyrex vial, compound 2 (70mg, 0.108mmol), NaHCO₃ (35mg, 0.129mmol) and 4-nitrophenyl chloroformate (25mg, 0.431mmol) were dissolved in anhydrous MeCN (3.2mL) and allowed to stir overnight at room temperature under N₂. After 24 hours, TLC shows formation of a new product. Water (2mL) was added and the reaction was allowed to continue for another 15 hours at which time TLC showed the reaction reached completion. The pH was adjusted to 5 with 0.1N HCl and the solution was evaporated under reduced pressure to remove excess MeCN. The reaction mixture was extracted with ethyl acetate (3 x 15mL) and the combined organic fractions were dried over MgSO₄ before concentrating under reduced pressure. The crude product was purified twice by column chromatography (0-50% ethyl acetate in dichloromethane) with a silica stationary phase to yield CO31 (25mg, 34.3%).
2.3 Synthesis of SF01

2.4 Scheme of SF01 synthesis

Figure 8: Synthesis of SF01
2.4.1 6-(4-iodophenyl)hexan-1-ol

Methyl 6-(4-iodophenyl)hexanoate, previously created for the synthesis of JZ01, (521mg, 1.57mmol) was dissolved in anhydrous DCM (6mL) under N₂. This solution was brought to -78°C before dropwise addition of 1.0M DiBAL in THF (5.5 mL, 5.5mmol). The reaction was allowed to react overnight while at room temperature. When TLC indicated the reaction to be complete, the reaction was quenched using saturated Rochelle’s salt in water and sodium sulfate was added to absorb remaining water. When the solution was clear, the organic layer was collected and concentrated under reduced pressure resulting in the crude product 1 (470mg, 98%). \(^1\)H NMR (400 MHz, Chloroform-d) δ 7.60 (d, 2H), 6.94 (d, 2H), 3.64 (t, J = 6.6 Hz, 2H), 2.57 (t, 2H), 1.60 (m, 4H), 1.37 (m, 4H).

2.4.2 Tert-butyl((6-(4-iodophenyl)hexyl)oxy)dimethylsilane

To compound 1 (470mg, 1.55mmol) and imidazole (313mg, 4.60mmol) in a Pyrex vial under N₂ is added a solution of TBS-Cl (470mg, 3.12 mmol) in anhydrous DCM (5mL). The solution was stirred over night at room temperature. The reaction
was washed with water (10mL) and extracted with DCM (3x15mL), and the combined organic extracts were dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by column chromatography on a silica stationary phase (0-10% ethyl acetate in hexanes) to yield 2 (420mg, 65%). ¹H NMR (400 MHz, Chloroform-d) δ 7.61 (d, 2H), 6.96 (d, 2H), 3.62 (t, J = 6.6, 2H), 2.57 (t, 2H), 1.58 (m, 4H), 1.36 (m, 4H), 0.93 (m, 9H), 0.08 (m, 6H).

2.4.3 Tert-butyl 2-(4-(3((tert-butyldimethylsilyl)oxy)hexyl)phenyl)ethynyl)-5-isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylphenoxy)acetate

To a solution of compound 2 (420mg, 1.00mmol) and tert-butyl 2-(4-(3-ethynyl-5-isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylphenoxy)acetate (455mg, 1.01mmol), previously created in the synthesis of JZ01, in piperidine (5mL) was added CuI (29mg) and Pd(PPh₃)₂Cl₂ (68mg). The vial was flushed with argon and stirred overnight at 45°C. The reaction mixture was combined with water (10mL) and the aqueous layer was extracted with ethyl acetate (3x15mL). The combined organic fractions were dried over MgSO₄, evaporated under reduced pressure and the residue was purified by column chromatography with a silica stationary phase (0-10% ethyl acetate in hexanes) to yield 3 (670mg, 77%). ¹H NMR (400 MHz, Chloroform-d) δ 7.43 (d, 2H), 7.17 (d, J = 8.0 Hz, 2H), 6.98 (s, 1H), 6.89 (s, 1H), 6.67 (s, 2H), 5.29
(s, 2H), 4.55 (s, 2H), 3.95 (s, 2H), 3.63 (m, 5H), 3.45 (p, J = 6.9 Hz, 1H), 2.63 (t, J = 7.7 Hz, 2H), 2.24 (s, 6H), 1.64 (t, J = 6.7 Hz, 1H), 1.52 (s, 12H), 1.38 (m, 4H), 1.21 (d, J = 7.0 Hz, 6H), 0.93 (s, 9H), 0.08 (s, 6H).

2.5 Proposed continuation of the synthesis of SF01

![Chemical Structure]

2.5.1 2-(4-(4-hydroxy-3-((4-(6-hydroxyhexyl)phenyl)ethynyl)-5-isopropylbenzyl)-3,5-dimethylphenoxy)acetic acid

To a solution of 3 (50mg, 0.0673mmol) in THF (3mL) was added 6N HCl (3mL). The reaction mixture was stirred under N₂ at 40°C overnight. THF was added as needed to maintain solubility. The reaction was poured over water (5mL) and extracted with ethyl acetate (3x15mL). The combined organic extracts were dried over MgSO₄, evaporated under reduced pressure, and the residue was purified by column chromatography with a silica stationary phase (0-10% MeOH/DCM) to yield 4 (18mg, 51%). ¹H NMR spectra were inconclusive so the next step is to try a modified version of this reaction using trifluoroacetic acid to deprotect 3 and obtain new ¹H NMR data.
2.5.2 SF01

To arrive at the final fluorescent probe, the deprotected scaffold molecule must be synthesized and identified; approximately 30mg will be needed. The primary alcohol group will then be oxidized to a ketone by Dess-Martin reduction. With this compound in hand, a final reductive amination using NaBH₃CN will couple the BODIPY fluorophore to create the final product, JZ01.

2.6 Preparation of TRβ and TRα expressing cells

2.6.1 Transfection strategy

To assay JZ01 and SF01 fluorescent probes, three separate transfections were performed. A first row of cells was transfected with TRα and mCherry expressing plasmids, the second row of cells was transfected with TRβ and mCherry expressing plasmids, a third row of cells was transfected with only the mCherry expressing plasmid, and a fourth control row was not transfected.

In all cases, mCherry was added to transfections to normalize the transfection efficiencies between wells. The mCherry fluorescent protein was chosen because its excitation and emission profile are separate from those of the fluorescein and BODIPY spectra (Figure 9). A control group, transfected with mCherry, was used to
account for any interactions that mCherry may have with the fluorescent probes used. A second control group was not transfected to account for any background fluorescence of the cell.

Figure 9: Excitation and emission spectra of JZ01, SF01, and mCherry

### 2.6.2 Transfection protocol

Lipofectamine 3000 obtained from ThermoFisher Scientific was used following manufacturer’s protocol to transfect the cells described. On the first day, human embryonic kidney cells (HEK 293) were introduced to a 24-well plate at a seeding number of $1.0 \times 10^5$. Cells were incubated in Dulbecco Modified Eagle Medium (DMEM) at 27°C in a CO$_2$ concentration of 5.0%. On day two, cells were observed to be 80% confluent.
To transfect on day two, 3.5μL from each stock of TRα and TRβ expressing plasmids were separately combined with 3.5μL of mCherry expressing plasmid and diluted in 150μL Opti-MEM medium with Lipofectamine P3000 Reagent. The mCherry control was transfected using 7.0μL of mCherry expressing plasmid as the amount of DNA used in each transfection must be held constant. This solution was then combined in a 1:1 ratio with Lipofectamine 3000 Reagent that had been diluted in 150μL of Opti-MEM medium and allowed to incubate at room temperature for fifteen minutes. Next, 50μL of this DNA-lipid complex was added to each respective well without changing the DMEM that cells were plated in. After eight hours of incubation with Lipofectamine, the media was aspirated and fresh media (DMEM) was added. Before assaying on the fourth day, minimal cell death was observed.

![Figure 10: Representation of cell transfection in 24 well plate with JZ01 concentration gradient](image)
2.7 Cellular fluorescent probe assays

Stock solutions (1000x) of JZ01 were created at molar concentrations of $1.0 \times 10^{-4}$, $5.0 \times 10^{-5}$, $1.0 \times 10^{-5}$, $1.0 \times 10^{-6}$, and $5.0 \times 10^{-7}$ in DMSO. To stain cells, 1.0μL of each stock solution was added to each 1.0mL well to ensure the final concentration of DMSO would be equal among all stained cells (1:1000). After introducing JZ01, the cells were incubated at 27°C in 5.0% CO$_2$ for one hour. After incubating for an hour, the media was aspirated and 0.5mL of Dulbecco’s Phosphate-Buffered Saline (DPBS) was gently added and aspirated. In the first trial, an additional 0.5mL of DBPS was added before visualizing cells. In a second trial, all media and DPBS were aspirated before visualizing. Cells were visualized immediately after rinsing.

2.7.1 Fluorescence Microscopy

Immediately after staining and rinsing, cells were visualized using traditional optical microscopy and fluorescence microscopy. When viewing JZ01, a filter specific to fluorescein was used (ex. 485nm, em. 535nm).

2.7.2 Fusion plate reader

A Perkin-Elmer fusion plate reader was used to quantify fluorescence produced by both the mCherry and the fluorescent probes separately. To detect for mCherry, cells were excited at 590nm and emission was determined at 620nm. To detect for JZ01, cells were excited at 485nm and emission was monitored at 535nm. This instrument detects and quantifies the overall fluorescence of each well using an average of five runs and assigns values of arbitrary fluorescence units to each well. Because units are arbitrary, values of wells within the same plate can be compared but values across plates cannot be compared.
Chapter 3

RESULTS

3.1 Synthesis of JZ01 and CO31

The first goal of this project involved resynthesizing JZ01 for collaborators currently using the probe to stain cells of interest in their studies. As the previous pool of JZ01 was depleted from its initial synthesis, it was resynthesized following the procedure described by Zheng. A total of 10mg of product was successfully synthesized, purified, and characterized. This sample was aliquoted to be used by collaborators and for use in later cell assays.

Additionally, CO31 was synthesized as a tool to enhance selective labelling in cells. Previous research shows that this compound forms selective interactions with TRα which would make it a useful tool in selectively labelling TRβ\(^{[13]}\). This synthesis followed a similar route to that described by Ocasio with several modifications. Because of poor yields, the protection and deprotection of alcohol and amine groups was forgone and the synthesis was simplified to three steps as opposed to seven. This scheme gave much greater yields and 25mg of product was successfully synthesized, purified, and characterized.

3.2 Synthesis of SF01

The final goal of the project was to create a novel fluorescent thyroid hormone receptor probe that had a greater utility than JZ01 for staining in cells. BODIPY was used in this synthesis because it has several desirable characteristics including an increased stability, reduced toxicity to cells, and a high fluorescence quantum yield in water\(^{[4,6]}\). This synthesis took advantage of the pool of intermediates created by the previous synthesis of JZ01. Additionally, this synthesis couples the fluorescent probe
in the last reaction which is ideal as even small quantities of fluorescent analogs are prohibitively expensive. At this point in the synthesis, 15mg of the deprotected scaffold has been synthesized and purified before characterization. Although $^1$H NMR showed characteristic peaks, neither this method or mass spectrometry gave a conclusive profile. After this intermediate has been thoroughly characterized, it will be oxidized by Dess-Martin reaction before being coupled with the BODIPY probe in a final, reductive amination step. After synthesis, this compound will be purified using HPLC and characterized with $^1$H NMR, $^{13}$C NMR, and mass spectrometry.

3.3 Staining with JZ01

3.3.1 Trial one

After plating and transfecting HEK 293 cells, a gradient of JZ01 from 0 to 100nM was used to stain. Cells were characterized in a solution of DBPS and it was observed that they began to detach from the plate; however, they were not aspirated off. Observing cells using fluorescence microscopy initially did not show any fluorescence of JZ01. After initially observing cells in DBPS, it was hypothesized that the background fluorescence of the solution may be masking signal from cells. To view cells with minimal interference, DBPS was aspirated off and cells were visualized a second time. Using a fluorescein filter, the synthesized JZ01 was observed to be localized in cells.

After visualizing cells, the 24-well plate was assayed using a fluorescence plate reader that quantified mCherry fluorescence and JZ01 fluorescence separately. In this trial, controls included untransfected cells, cells only transfected with mCherry, and unstained cells. Fluorescence data are reported in Tables 1 and 2.
Table 1: mCherry fluorescence of cells (trial 1)

<table>
<thead>
<tr>
<th></th>
<th>100nM</th>
<th>50nM</th>
<th>10nM</th>
<th>1nM</th>
<th>0.5nM</th>
<th>0nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trα + mCherry</td>
<td>123293</td>
<td>102385</td>
<td>79153</td>
<td>76366</td>
<td>73362</td>
<td>75895</td>
</tr>
<tr>
<td>Trβ + mCherry</td>
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<td>79918</td>
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<td>74723</td>
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<tr>
<td>mCherry</td>
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<td>77027</td>
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<td>77538</td>
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<td>91223</td>
<td>83458</td>
<td>87976</td>
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Table 2: JZ01 fluorescence of cells (trial 1)

<table>
<thead>
<tr>
<th></th>
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<th>50nM</th>
<th>10nM</th>
<th>1nM</th>
<th>0.5nM</th>
<th>0nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trα + mCherry</td>
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<td>54527</td>
<td>54042</td>
<td>52202</td>
<td>51894</td>
</tr>
<tr>
<td>Trβ + mCherry</td>
<td>99899</td>
<td>180745</td>
<td>69249</td>
<td>56261</td>
<td>55237</td>
<td>51720</td>
</tr>
<tr>
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<td>57977</td>
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<td>64133</td>
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<td>54174</td>
<td>52790</td>
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Trends in the data collected in trial one show several concerning discrepancies including little difference in the mCherry fluorescence between mCherry transfected cells and untransfected cells. This may point to either a flawed transfection or a loss in cells due to detachment. Additionally, no concrete trend can be distinguished between cells expressing TRα, TRβ, and control cells after staining with JZ01 (see Figure 11). This effect may also be due in part to assaying cells while in solution of DPBS.

To analyze the fluorescence data collected, several factors and their controls must be considered. First, the background fluorescence of the cells was accounted for by subtracting the fluorescence of unstained samples. Essentially, the fluorescence of the last well in each row was subtracted from each well preceding it. This yielded the total fluorescence from localized JZ01. Next, to account for transfection efficiencies in each well, JZ01 fluorescence of the well was divided by its corresponding mCherry fluorescence. This ratio normalized for the efficiency of each transfection, well-to-well.
This relative fluorescence value was plotted as a function of JZ01 concentration to allow for the comparison of cells expressing TRα, TRβ, and control cells. As the probe had been characterized in the past to preferentially label TRβ, one would expect to see the greatest fluorescence in TRβ expressing cells, moderate fluorescence in TRα expressing cells (as it binds JZ01 to a lesser extent), and little fluorescence in cells only expressing mCherry. In trial one, this was not the case as no trends were observed.

Figure 11: Relative fluorescence of cells at increasing JZ01 concentrations (trial 1)

3.3.2 Trial two

The experimental procedure was modified in trial two to account for the possible shortcomings of the previous assay. Cells were transfected in the same fashion with the same plasmids. After incubating with JZ01 in this trial, media was aspirated and cells were rinsed with 0.5mL of DPBS again. This time, however, the
DPBS was aspirated and cells were observed with no added solution (without media, DPBS, etc.). When observed using optical microscopy, there was no sign of cell detachment. Additionally, fluorescence microscopy showed the fluorescence characteristic of JZ01 localized in cells that were stained at higher concentrations (50nm, 100nm). Again, the fluorescence plate reader was used to quantify intensity and the collected data are reported in Tables 3 and 4.

Table 3: mCherry fluorescence of cells (trial 2)

<table>
<thead>
<tr>
<th></th>
<th>100nM</th>
<th>50nM</th>
<th>10nM</th>
<th>1nM</th>
<th>0.5nM</th>
<th>0nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trα + mCherry</td>
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<td>126338</td>
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<tr>
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<td>125813</td>
<td>121929</td>
<td>127751</td>
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<tr>
<td>mCherry</td>
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<td>131560</td>
<td>130652</td>
<td>130195</td>
<td>127413</td>
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</tbody>
</table>

Table 4: JZ01 fluorescence of cells (trial 2)

<table>
<thead>
<tr>
<th></th>
<th>100nM</th>
<th>50nM</th>
<th>10nM</th>
<th>1nM</th>
<th>0.5nM</th>
<th>0nM</th>
</tr>
</thead>
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<tr>
<td>Trα + mCherry</td>
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<td>82974</td>
<td>82626</td>
<td>81650</td>
<td>81316</td>
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</table>
Table 5: Relative fluorescence values (trial 2)

<table>
<thead>
<tr>
<th></th>
<th>Con (nM)</th>
<th>Fluorescence (corr.)</th>
<th>Con (nM)</th>
<th>Fluorescence (corr.)</th>
</tr>
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<td><strong>TRα</strong></td>
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<td></td>
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<td>-0.001607493</td>
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<tr>
<td><strong>mCherry</strong></td>
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In trial two, the same corrections were used to account for the fluorescence readings of each well; first the fluorescence of unstained cells was subtracted from the stained fluorescence and then the fluorescence of each well was divided by its mCherry fluorescence value. Relative fluorescence was plotted as a function of concentration of JZ01. In this trial, it appears that the modified staining procedure helped to achieve more realistic results. As expected, TRβ expressing cells show the greatest fluorescence while TRα expressing cells show a moderate response and mCherry expressing control cells showed little response to the fluorescent probe.
3.3.3 Future trials

The first trials of JZ01 staining afforded several pieces of important information. First, the resynthesized JZ01 has the same characteristics as the JZ01 previously synthesized by Zheng. This probe is effectively localized in cells, fluoresces at a wavelength characteristic of fluorescein, and shows preferential binding to TRβ over TRα over cells with no transfected TR. Secondly, at concentrations of 50-100nM there is a large and distinguishable difference in JZ01 staining between TR phenotypes. These determinations are single data points so it is important to confirm the trend with triplicate analyses.

The next step in this process is assay cells again at concentrations of 50nM and 100nM JZ01 to obtain triplicate results and establish a statistical difference between
the cells stained. Once these conditions are analyzed, the concentration that provides the clearest results will be used in assays testing the utility of CO31. Holding the concentration of JZ01 constant, a concentration gradient of CO31 will be used to determine its effect on the fluorescent labelling of thyroid hormone receptors TRα and TRβ. Similarly, after its synthesis has been completed, SF01 will be assayed side-by-side with JZ01 to determine its effectiveness in staining cells at varying concentrations.

By adjusting the experimental design used, an optimized methodology was created to test the products of this project’s synthesis. The transfected cells were most effectively incubated with JZ01 in media and fluorescence was most accurately determined in wells containing cells alone (no DMEM or DPBS). Future assays will determine the effectiveness of CO31 and SF01 using this modified procedure.
Chapter 4
DISCUSSION

An understanding of thyroid hormone receptors is essential to human health as they regulate the cell cycle and TRβ specifically is known to have tumor suppressing qualities [9]. Hypothyroidism, or a deficiency in thyroid function, is known to increase metastases in cancers and TRβ is often mutated given its location on chromosome 3 [16]. Because of its propensity to mutate, a lack of TRβ function has been linked to thyroid, lung, and breast cancer while 40.9% of ccRCC tissues have been found to contain mutations in TRβ. This overwhelming occurrence is worrying considering the tyrosine kinase inhibitors prescribed to ccRCC patients tend to cause side effects in patients that include impairment of thyroid function [5]. The need to further explore the relationship between TRs and occurrence of cancer relies on the development of analytical techniques capable of characterizing these receptors in cells.

By developing and improving fluorescent thyroid hormone receptor probes along with other techniques to improve their efficiencies, the ability to analyze TR in live cells is becoming more approachable. This advantage of analyzing TR in live cells is unique to fluorescent thyroid hormone receptor probes as antibodies are only truly useful in characterizing dead cells and tissues. Current research takes advantage of these fluorescent probes to selectively bind cancer precursor cells and separate them from surrounding tissues. After incubating cells with JZ01, FACS separates cells based on the fluorescence attributed to JZ01. After sorting, this probe can then be removed from the living cells by diffusion, leaving an aliquot of cells overexpressing TRβ for further research.
While JZ01 and CO31 were successfully synthesized, their relationship inside cells will require more exploring. The resynthesized JZ01 tested displayed the same characteristics of the JZ01 previously synthesized by Zheng as the probe localized in cells and was subtype selective for TRβ expressing cells. The methodology of this staining was originally problematic, but it was found that cells were ideally incubated with JZ01 in DMEM media and subsequently observed out of solution. This same methodology will be used in future assays after statistical difference is established in the concentrations of JZ01 tested. Cells will then be stained with a constant concentration of JZ01 while a gradient of CO31 is used to modulate TRα vs TRβ selectivity. An effective assay would show a more dramatic response to CO31 in TRα cells than TRβ cells making the probe more selective for the TRβ subtype at equivalent concentrations.

The final goal in this project is to create the novel probe, SF01. At this point, there remains two steps left in the synthesis of this new fluorescent probe. Future plans include the completion of this probe along with testing its application in staining. When staining with SF01, visually inspecting cells would determine if it is localized in cells as is the case with JZ01. Additional tests will include assaying with the fluorescent plate reader used in JZ01 staining using a similar concentration gradient among transfected cells. This probe would also be compared side by side to JZ01 to determine if it is able to efficiently stain cells at concentrations similar to JZ01.
REFERENCES


Appendix A

$^1$H NMR and $^{13}$C NMR Spectra

Figure 13: $^1$H NMR of JZ01

Figure 14: $^{13}$C NMR of 2-amino-3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)propenamide
Figure 15: $^{13}$C NMR of CO31

Figure 16: $^1$H NMR of CO31
Figure 17: $^1$H NMR of 6-(4-iodophenyl)hexan-1-ol

Figure 18: $^1$H NMR of tert-butyl((6-(4-iodophenyl)hexyl)oxy)dimethylsilane
Figure 19: 
$^1$H NMR of Tert-butyl 2-(4-(3-((4-(6-((tert-butyldimethylsilyl)oxy)hexyl)phenyl)ethynyl)-5-isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylphenoxy)acetate

Figure 20: 
$^1$H NMR of 2-(4-(4-hydroxy-3-((4-(6-hydroxyhexyl)phenyl)ethynyl)-5-isopropylbenzyl)-3,5-dimethylphenoxy)acetic acid