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Characterization of Ligands for Thyroid Receptor Subtypes and their Interactions with Co-Regulators

September 2008

Characterization of Ligands for Thyroid Receptor Subtypes and their Interactions

with Co-Regulators

by

Elizabeth J. Koury

A Thesis

Presented to the Graduate and Research Committee

of Lehigh University

in Candidacy for the Degree of

Master of Science

in

The Department of Chemistry

Lehigh University

15 August 2008

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Abstract

Thyroid hormone receptors (TRs) are nuclear receptors that are activated by thyroid hormone ligands and co-regulator proteins. Two receptor subtypes, TRα and TRβ, have been identified and are implicated in numerous physiological functions. However, due to the lack of TR-specific ligands, full characterization of these receptors, their specificity of function and specific mechanisms of action is largely unknown. Therefore, bioassays that can identify TR-selective ligands are essential. A biochemical assay was developed for the purpose of compound library screening and a cell-based assay was developed to predict endogenous ligand-receptor specificity. Following assay optimization, 5 known TR ligands were evaluated to characterize their selectivity and interaction with the TR subtypes. In the biochemical assay, the rank order of potency was similar in the presence of either SRC1-2 or NCoR, with T3 and Triac potencies greater in the presence of NCoR. The potency of Tetrac remained constant regardless of co-regulator. The T4 and rT3 ligands demonstrated selectivity for TRα versus TRβ subtype. Conversely, in the cell-based assay all ligand potencies decreased and lacked receptor selectivity, although the rank order potencies remained the same. The utility of using both the biochemical and the cell-based assays in TR drug discovery will be discussed.

1. Introduction

Nuclear receptors (NRs) are a large family of ligand-activated transcription factors that regulate the expression of target genes. The functionality of NRs is dependent on the ligand bound, as well as its interaction with components of transcriptional machinery [1]. Typical ligands involved in activation of NRs include steroid and thyroid hormones, retinoids and vitamin D. Once the ligand is bound to the NR, the NR and ligand complexes with additional factors, eventually binding to its associated hormone response element which is located in the promoter region of the target gene **(Fig.1)**.
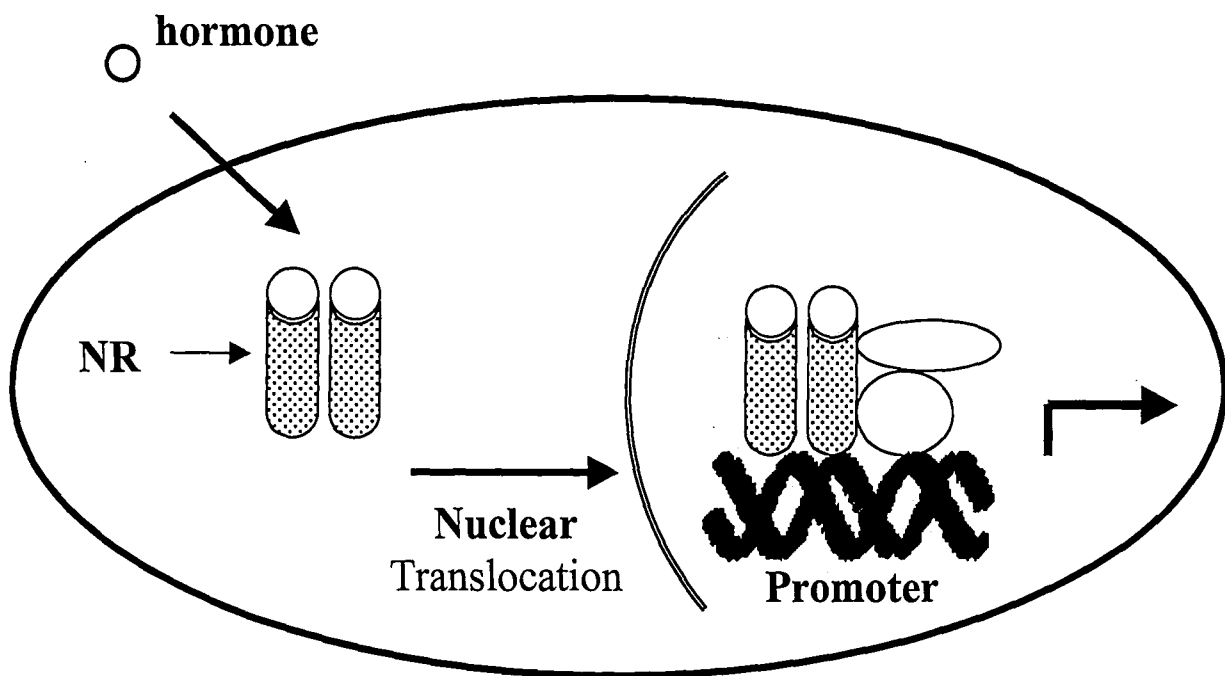
Fig. 1. Ligand bound nuclear receptors (NR) translocate into the nucleus where they complex with co-regulators and bind to its associated response element.

This initiates transcriptional activation by transmitting signals to the basal transcriptional machinery, through protein-protein interactions. [2]. Chromatin facilitates the assembly of this basal transcriptional machinery and increases the rate of transcription.

Nuclear receptor co-regulators are required for efficient transcriptional regulation. These are cellular factors recruited by NRs that complement their function as mediators of the cellular response to endocrine signals. Co-regulators are rate limiting for NR activation and repression. Co-activators are molecules that enhance the transactivation of nuclear receptors. Transactivation is an increased rate of gene expression triggered by endogenous proteins. Conversely, co-repressors are factors that interact with NRs which lower the transcription rate at their target genes [3]. It is hypothesized that NRs switch in a ligand dependent manner, between binding a multi-component co-repressor and binding a co-activator complex. The co-repressor complex contains histone deacetyltransferase activity, while the co-activator complex contains histone acetyltransferase activity and is further influenced by additional signal transduction pathways [4].

Thyroid hormone receptors (TR) are members of the NR superfamily that is activated by the binding of thyroid hormones. The binding of the ligand causes recruitment of the co-activator complexes that increase histone acetylation and recruit RNA polymerase II to activate transcription [5]. The 3,5,3'-triiodothyronine (T3) ligand is the most active form of thyroid hormone in target tissue. Activation or suppression of TR affects numerous critical physiological processes including development, growth and metabolism in higher organisms [6]. Investigation into the cause of cretinism and myxedema, led to the discovery that these diseases were specifically due to the loss of thyroid function. More importantly, this investigation led to the discovery of T3 and L-

thyroxine or T4, the most potent of the thyroid hormones [7]. The ligand T4 is a

prohormone which is metabolized to the active hormone T3 by either Type I 5'-

deiodinase or Type II 5'-deiodinase [8].

TR is encoded by two separate genes, TRα and TRβ. The first subtype, TRα, was

originally cloned from chick embryo and mapped to human chromosome 17 [9]. The

TRβ subtype was cloned from human placenta and found on chromosome 3 [10]. Each

subtype has multiple isoforms, TRα1, TRα2, TRα3 and TRβ1 and TRβ2.

The primary function of a TR as a transcription factor, is to regulate target gene

expression directly through DNA response elements and more specifically the T3

response element (TRE). The TR ligand has the ability to bind TREs constitutively

independent of ligand occupancy. TRs bind TRE as heterodimers with retinoid X

receptor (RXR) **(Fig. 2)**. This heterodimerization is thought to enhance the affinity of
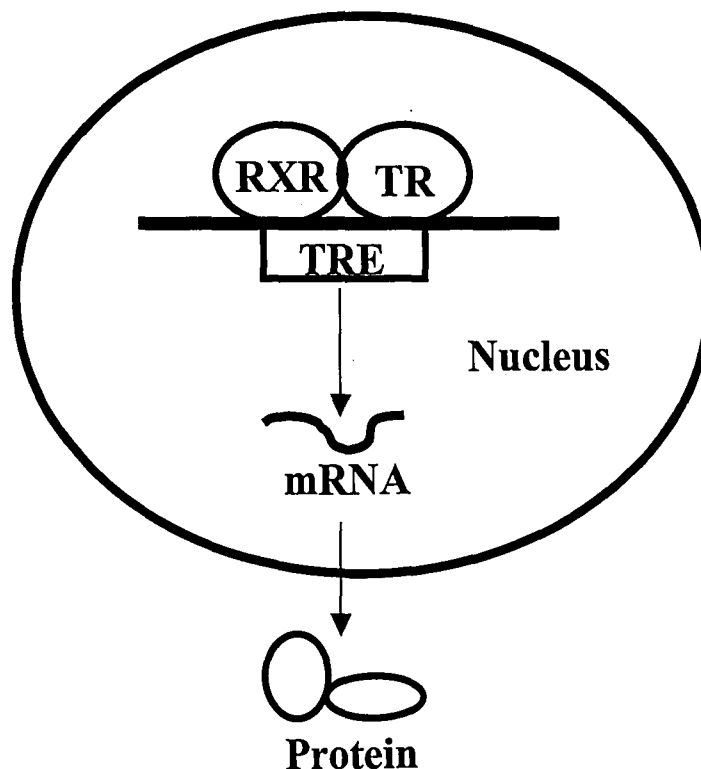
Fig. 2. Thyroid receptors (TR) heterodimerize with retinoid X receptor (RXR), enhancing binding of TR to the thyroid response element (TRE).

DNA binding and provide increased target gene specificity [11]. Distinct mechanisms are used by the various isoforms to regulate this heterodimerization. However, when ligand is bound, it initiates a conformational change in the TR, activating transcription of its target gene. Conversely, an unliganded TR generally represses basal transcription. The novelty to TR as well as all other liganded nuclear receptors, is that transcriptional activation is mediated by co-activator and co-repressor protein interactions.

Many NRs, including TR, contain a highly conserved subregion within the carboxy terminus of the ligand-binding domain (LBD). This region is necessary for transcriptional activation and is termed activation function 2, or AF-2 region **(Fig. 3)** [4].

| N | A/B | DBD | Hinge | LBD | | F | C |

AF-1   AF-2
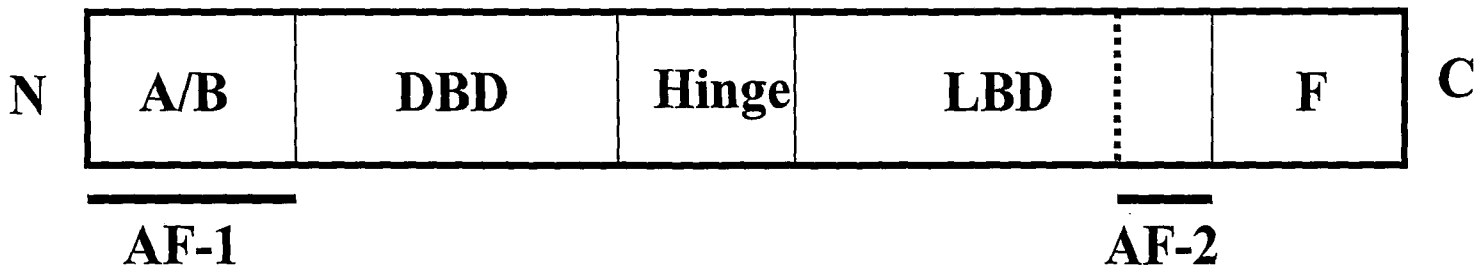
Fig. 3. Nuclear receptors can be divided into six subregions. Region A/B contains the ligand independent transactivation domain, AF-1. DBD is the DNA binding domain, followed by a hinge region which is part of the LBD, or ligand binding domain. AF-2 is the ligand dependent transactivation domain, which is also contained within the LBD.

Also, most NRs contain a non-conserved activation domain in the amino terminal referred to as the AF-1 region. This domain is highly divergent between the TRα and TRβ isoforms, suggesting that these isoforms play different roles in transcriptional regulation. Because this amino terminal domain is not required for T3-dependent transcriptional activation by rat TRβ1, it suggests that this domain may not be essential

for TR function [11]. The AF-1 domain functions to modulate both ligand-dependent activation as well as ligand-independent interaction with co-repressors.

On the surface of most co-activators there is a highly conserved region referred to as the LXXLL motif. These are helical domains with amphipathic characteristics. There is a comparable motif in the AF-2 region of NRs, suggesting that initial contact between activated NRs and co-activators is highly reliant on this LXXLL motif. More specifically, crystallographic evidence suggests that there is a ligand-dependent shift in helix 12, a critical helix in the AF-2 region which creates a thermodynamically secure environment for the LXXLL motif [4] [12].

The interaction of TR and co-repressors, resides in a separate distinct region of the LBD than that of receptor/co-activator interactions. The functionality of these co-repressors is separate from the AF-2 domain. A core co-repressor complex contains more than seven polypeptides, including the histone deacetylases referred to as HDAC1 and HDAC2. It is suggested that these transcription factors that function as repressors, employ a common mechanism involving the recruitment of multi-component complexes. These complexes have histone deacetylase activity and re-establish a repressive chromatin state, therefore, down-regulating basal transcription as chromatin plays an important role in regulating the basal activity of many promoters.

To date, T3 has been almost exclusively used as the tool compound in identifying the mechanism of action of thyroid hormones. However, derivatives of T3 such as triiodothyroacetic acid (TriAc), 3,3',5'-triiodothyronine (rT3), and also L-thyroxine, or T4, and its derivative tetraiodothyroacetic acid (Tetrac), have not been evaluated for their effects on TRα and TRβ in one assay format [13]. It is our intention to evaluate the

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effects of these TR agonist compounds in combination with the co-activator peptide SRC1-2 and the co-repressor peptide NCoR on both the TRα and TRβ subtypes.

SRC-1 is a steroid receptor co-activator, the most common, that contains three LXXLL motifs and is essential for T3 function. It has been found that mice without functional SRC-1 are T3 resistant [11]. The NCoR co-regulator is a co-repressor that has two interaction sites. This correlates with the finding that two LBDs from two NRs are required for functional interaction with co-repressors on DNA. The TRs have a CoR box in the hinge region of helix 1 which is required for interaction with co-repressors.

A technology referred to as the AlphaScreen, is an association bioassay ideally suited for studying co-recruitment. This bioassay employs glutathione-S-transferase (GST) coated beads that complex with protein, ligand and co-peptide **(Fig. 4)**.

$^1O_2$

Excitation
680 nm

Emission
520-620 nm

AlphaScreen Donor Bead   A   B   AlphaScreen Acceptor Bead
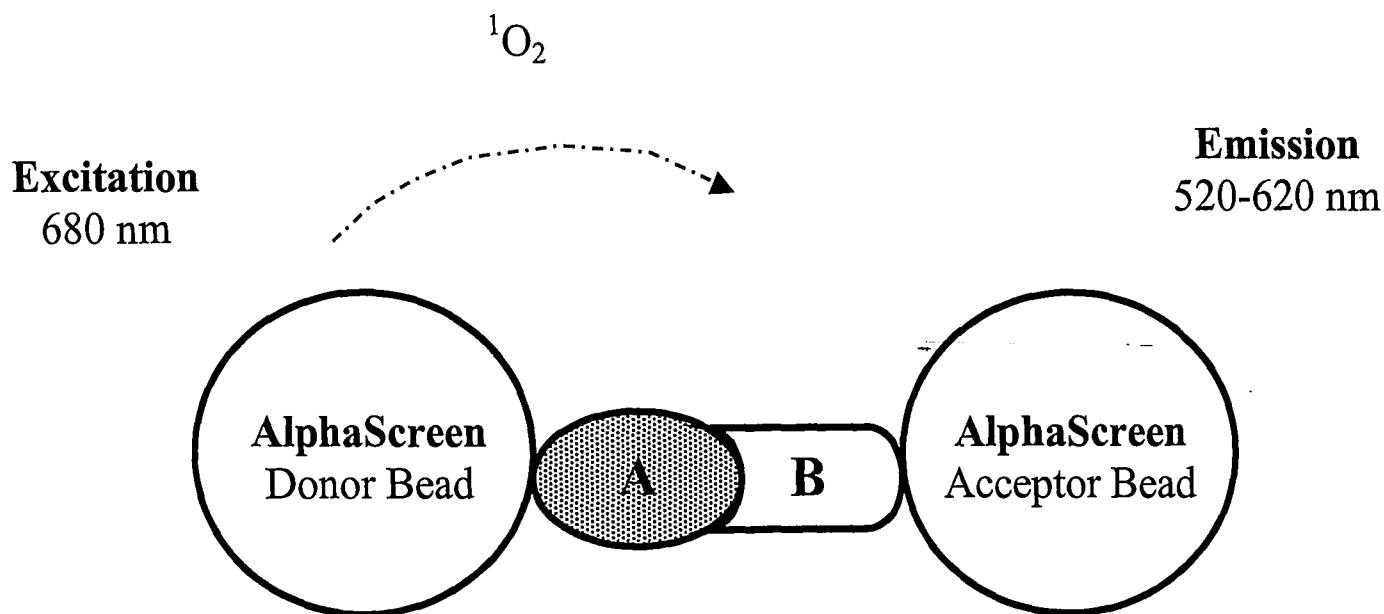
Fig. 4. Thyroid hormone AlphaScreen biochemical assay. In the presence of ligand, the biotinylated co-regulator, **A**, binds to the donor bead and fuses with the TR-LBD-GST, **B**, which is bound to the acceptor bead. When brought into close proximity, these beads initiate a cascade of chemical reactions resulting in the emission of light at 520-620 nm.

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This assay was developed and utilized as the biochemical assay to investigate receptor, ligand and co-regulator interactions. In this study, five compounds, T3, T4, Triac, Tetrac and rT3, will be characterized using this assay. Additionally, a one hybrid cell-based assay was developed and used to investigate the functional response of transiently transfected COS-7 cells to these ligands and enable the ability to measure transactivation. The intention of this study is to use a biochemical assay in conjunction with a cell-based assay to evaluate data between assay platforms using 5 TR ligands. This will provide insight into the functions of the TRs and the co-activators and co-repressors in the presence of various thyroid hormones and their ligand derivatives.

2. Materials and Methods

2.1. Chemicals and Reagents

The thyroid hormone agonists 3,5,3'-triiodothyronine (T3), triiodothyroacetic acid (TriAc), 3,3',5'-triiodothyronine (rT3), L-thyroxine, (T4), and tetraiodothyroacetic acid (Tetrac), were obtained from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) for compound dissolution, as well as bovine serum albumin (BSA) was also obtained from Sigma-Aldrich (St. Louis, MO). The buffer components used in the AlphaScreen included potassium chloride (KCl) and Tris, HCl, pH 8.0 which were obtained from Invitrogen Corporation (Carlsbad, CA) and dithiothreitol (DTT) which was obtained from Millipore (Billerica, MA). The TRα-LBD and TRβ-LBD proteins were obtained from Invitrogen Corporation (Carlsbad, CA). The steroid receptor co-activator peptide (SRC1-2) and the nuclear receptor co-repressor peptide (NCoR) used in

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these studies were obtained from Anaspec (San Jose, CA). The Glutathione-S-Transferase (GST) AlphaScreen beads were obtained from Perkin Elmer (Waltham, MA).

The Gateway pENTR1A vector used to make the constructs for transfection was obtained from Invitrogen Corporation (Carlsbad, CA). The reagents used in the transfection procedure included phenol red free (PRF) Dulbecco's Modified Eagle Medium (DMEM), charcoal stripped fetal bovine serum, GlutaMAX, sodium pyruvate and Opti-MEM, were all obtained from Invitrogen Corporation (Carlsbad, CA). FuGENE 6 was obtained from Roche Diagnostics Corporation (Indianapolis, IN) and the luciferase cell culture lysis reagent for detection in the one-hybrid assay was obtained from Promega (Madison, WI).

2.2. Cell Culture

The COS-7 cells, African green monkey kidney fibroblasts, (ATCC, Manassas, VA) were cultured in DMEM with GlutaMAX, 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin Invitrogen Corporation, Carlsbad, CA. The cells were maintained at 37°C, 5% CO_2 and passaged every 72 h at a ratio of 1 mL of cell suspension to 10 mL of media. On the day of transfection, the cells were resuspended at 100,000 cells per mL in PRF-DMEM containing 10% charcoal stripped fetal bovine serum, 2 mM GlutaMax, and 1 mM sodium pyruvate. The cells were plated in 100 μL aliquots, in a 96-well plate to reach a final cell density of 10,000 cells in each well.

2.3. Co-recruitment Assay

The AlphaScreen technology and methodology has been described previously [14]. A modified version of these methods was optimized establishing the following assay conditions: 5 nM purified GST labeled TRα-LBD (amino acid sequence 148-410) or TRβ-LBD (amino acid sequence 202-461), 10 nM SRC1-2 or NCoR biotinylated peptide and a 1:250 dilution of Glutathione-S-Transferase AlphaScreen beads. The combination of these components will later be referred to as the protein/bead/peptide mixture. The sequences for SRC1-2 and NCoR peptides were Biotin-HSSLTERHKILHRLLQEGSPSDITT **(Fig. 5)** and Biotin-KKTHRLITLADHICQIITQDFARN, respectively (Anaspec, San Jose, CA).

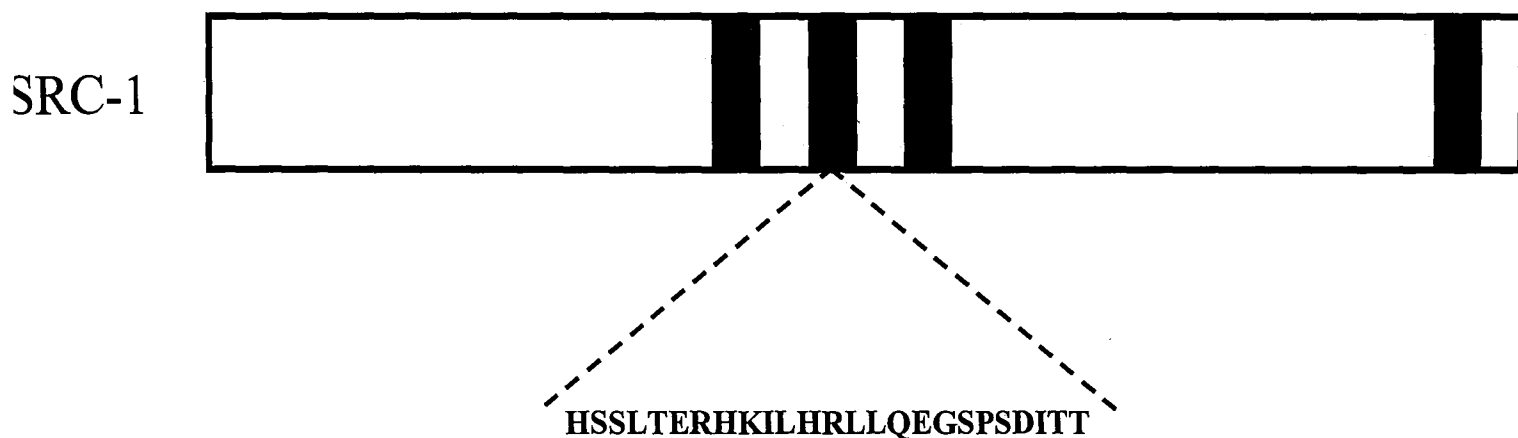**Fig. 5.** The co-activator peptide SRC-1 contains three LXXLL binding motifs within the nuclear-receptor interaction domain. The fragment of the SRC-1 peptide that was used in the biochemical assay was the SRC1-2 LXXLL binding motif.

The assay buffer contained 50 mM KCl, 50 mM Tris, pH 8.0, 2 mM DTT and 0.1% BSA. The plates used for this bioassay were 384-well Proxiplates (Perkin Elmer, Waltham,

MA). In order to establish optimal assay conditions, appropriate protein concentrations and incubations times were identified. In the experiments that were performed to determine the optimal protein concentration, wells containing 1, 3, 5 and 10 nM of protein were tested in the assay in the presence of the T3 ligand. In determining the optimal incubation time, the assay conditions were run in the presence of T3 at 1, 2, 4, 5 and 6 h.

Compounds were diluted using an 8-point serial dilution by half log increments. The starting concentration for T4, Tetrac and rT3 was 1 mM, while the starting concentration for Triac and T3 was 10 μM. The compound dilutions were made initially in a Matrix 384-well polypropylene plate (Thermo Fisher Scientific, Hudson, NH), in 100% DMSO using the Biomek FX automated pipetting system (Beckman-Coulter, Fullerton, CA). From the initial compound dilution plate, the compounds were subsequently diluted 10-fold into 100% DMSO, followed by a 2.5-fold dilution into assay buffer. These dilutions resulted in the highest concentrations of T4, Tetrac and rT3 being 40 μM and Triac and T3 being 400 nM. An aliquot of 5 μL of the serially diluted compounds was added to the Proxiplate, followed by an additional 5 μL of assay buffer. Finally, 10 μL of the protein/bead/peptide mixtures were added to the assay plate for a total volume of 20 μL. The highest concentration in the serial dilution of T4, Tetrac and rT3 in the assay was 10 μM whereas, the highest concentration of Triac and T3 in the assay was 100 nM. The final DMSO concentration was 9% DMSO in the assay. The addition of the beads was done under green light as the GST-beads are light sensitive. The plates were kept in the dark at room temperature for 2 h and were then counted on the EnVision microplate analyzer (Perkin Elmer, Waltham, MA).

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2.4. One-Hybrid Cell-Based Assay

Constructs in which human TRα-LBD and TRβ-LBD domains were cloned into the Gateway pENTR1A vector (Invitrogen Corporation, Carlsbad, CA) were used. The GAL4 DNA binding domain was incorporated into these constructs. The LBDs were of the same amino acid sequences as those obtained from Invitrogen. COS-7 cells were suspended at 100,000 cells/mL in culture media composed of PRF-DMEM, 10% charcoal stripped fetal bovine serum, 2 mM GlutaMAX and 1 mM Sodium Pyruvate. The cells were then aliquoted in a 100 µL volume into each well of a 96-well Polystyrene CulturPlate (Perkin Elmer, Waltham, MA). This results in a final cell density of 10,000 cells per well. FuGENE 6 transfection reagent was used according to the supplier's recommendations (Roche, Indianapolis, IN). This transfection methodology has been previously described [15,16] with the following modifications. The GAL4/TR-LBD plasmid and the Luciferase plasmid were added in a volume that equals approximately a final concentration of 0.05 µg per well. The plate is then incubated at 37°C, 5% CO_2 for 5 h. Following transfection, compounds were serially diluted in 10% DMSO/media, with starting concentrations for T3 and Triac being 100 µM and rT3, Tetrac and T4 being 1 mM. Series of dilutions of T3 and Triac starting at 10 µM and rT3, Tetrac and T4, starting at 100 µM were added to the plate containing cells and media at a 1% DMSO final concentration. The plates were incubated at 37°C, 5% CO_2, overnight, for approximately 17 h. The following day, the media was removed and a 25 µL aliquot of lysis reagent was added to each well and the plate was placed on a shaker for 10 minutes to lyse the cells. A 50 µL aliquot of the luciferase assay reagent was added to each well,

and the luciferase enzyme activity was measured using the Victor plate reader (Perkin Elmer, Waltham, MA).

2.5. Statistical Analysis

The EC_{50} values, which represent half of the maximal effective dose of the compound, were determined by using nonlinear regression sigmoidal dose response analysis in SAS-Excel using the equation $Y = Min+(Max-Min)/[1+exp(Hillx(log(EC_{50})-log(X)))]$. The fold difference in the AlphaScreen was determined by dividing the maximum counts per second (cps) values by the background cps values. The fold difference in affinities of the compounds between the biochemical assay and the cell-based assay was determined by dividing the EC_{50} value in the cell-based assay by the EC_{50} value determined in the biochemical assay.

3. Results

3.1. Biochemical Assay Optimization

In order to optimize the AlphaScreen biochemical assay conditions for this study, it was necessary to determine the optimal amount of TRα and TRβ protein as shown in **Fig. 6A** and **6B**. It was apparent that 1 nM of protein was an insufficient amount, particularly when using TRα protein. As 3 nM protein did demonstrate sufficient efficacy, it may be the lower limit of acceptability as anything below this concentration may compromise the consistency and reproducibility of the data. Therefore, 5nM was the protein concentration used in these studies.
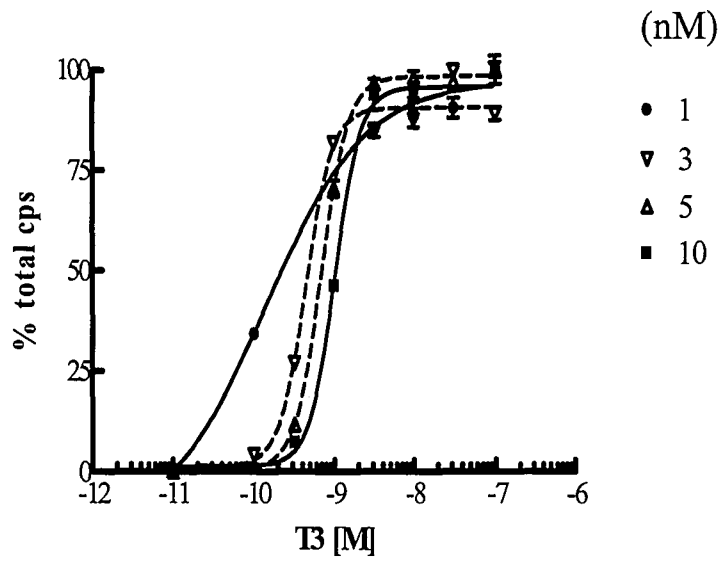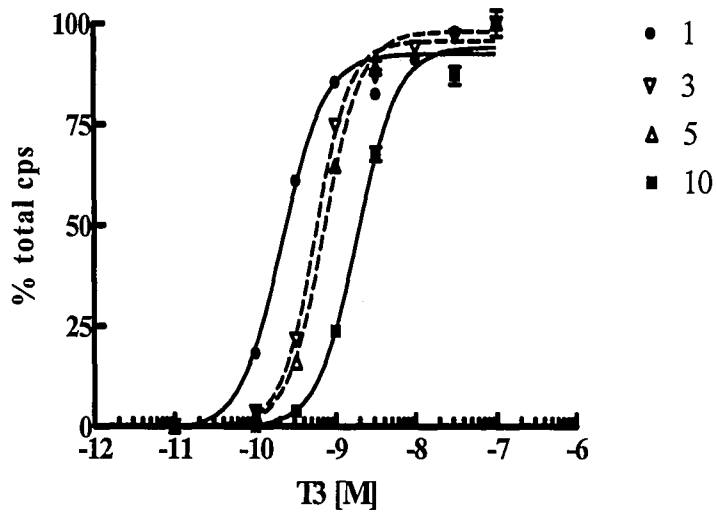
Fig. 6. Logistic concentration curves of the thyroid hormone T3 in the biochemical assay, using varying amounts of TRα protein (Panel A) and TRβ protein (Panel B).

In addition, it was necessary to determine the appropriate incubation time for the biochemical assay as demonstrated in **Fig. 7A** and **7B**. Logistic concentration curves of thyroid hormone T3 were tested using 5 nM of both the TRα and TRβ proteins.

A

B
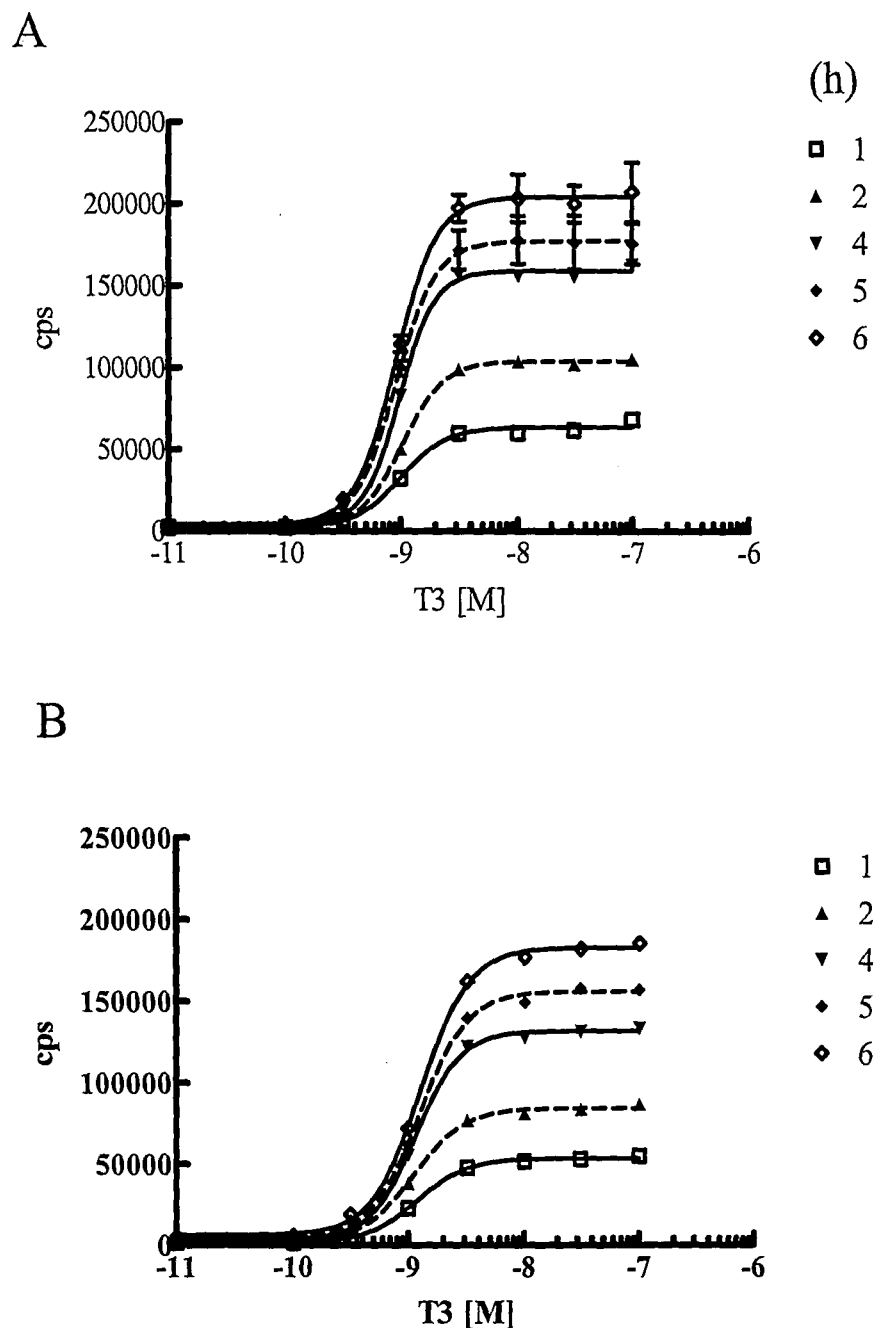
Fig. 7. Change in thyroid hormone T3 logistic concentration curves in the biochemical assay over time, using 5 nM TRα protein (Panel A) or TRβ protein (Panel B).

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Each concentration curve was incubated for either 1, 2, 4, 5 or 6 h. The fold changes were determined at each of these time points by dividing the total cps of each curve by the background cps for each curve as seen in **Tables 1A** and **1B**. From these results, it was determined that 2h was the most suitable incubation time in that consistency in data was observed and the length of the incubation is more amenable to compound screening.

Table 1A. Determination of incubation time in the biochemical assay, using thyroid hormone T3 and TRα protein.

Time (h)	EC$_{50}$'s (nM $\pm$ SEM)	Total cps	Background cps	Fold difference
1	1.0 $\pm$ 0.12	68126	2102	32
2	1.0 $\pm$ 0.07	104738	1405	75
4	0.98 $\pm$ 0.10	162324	3729	44
5	0.91 $\pm$ 0.15	175354	4048	43
6	0.91 $\pm$ 0.14	206775	3373	61

Graphic depiction of logistic concentration curve (Fig. 6; Panel A)
T3, thyroid hormone.
TRα, thyroid hormone alpha receptor.
EC$_{50}$ values, concentrations that elicits half the maximal response of T3.
Total cps, signal achieved in the presence of the maximum concentration of T3, 100nM.
Background cps, signal achieved in the presence of 9%DMSO in assay buffer.
Fold difference, total cps/background cps.
h, hours.

Table 1B. Determination of incubation time in the biochemical assay, using thyroid hormone T3 and TRβ protein.

Time (h)	EC$_{50}$'s (nM $\pm$ SEM)	Total cps	Background cps	Fold difference
1	1.2 $\pm$ 0.08	55190	1605	34
2	1.1 $\pm$ 0.09	86929	1457	60
4	1.2 $\pm$ 0.07	133656	2627	50
5	1.2 $\pm$ 0.08	157444	3456	46
6	1.3 $\pm$ 0.09	185744	5197	36

Graphic depiction of T3 logistic concentration curve (Fig. 6; Panel B)
T3, thyroid hormone.
TRβ, thyroid hormone beta receptor.
EC$_{50}$ values, concentrations that elicits half the maximal response of T3.
Total cps, signal achieved in the presence of the maximum concentration of T3, 100nM.
Background cps, signal achieved in the presence of 9%DMSO in assay buffer
Fold difference, total cps/background cps.
h, hours.

Finally, it was necessary to determine the maximum concentration of DMSO that could

be tolerated in the biochemical assay. In order to do so, logistic concentration curves of

T3 were tested at varying concentrations of DMSO. Data from 0% and 10% DMSO are

illustrated in **Fig. 8**. It was apparent that the biochemical assay could tolerate ligands

solubilized in up to 10% DMSO without compromising the integrity of the data.
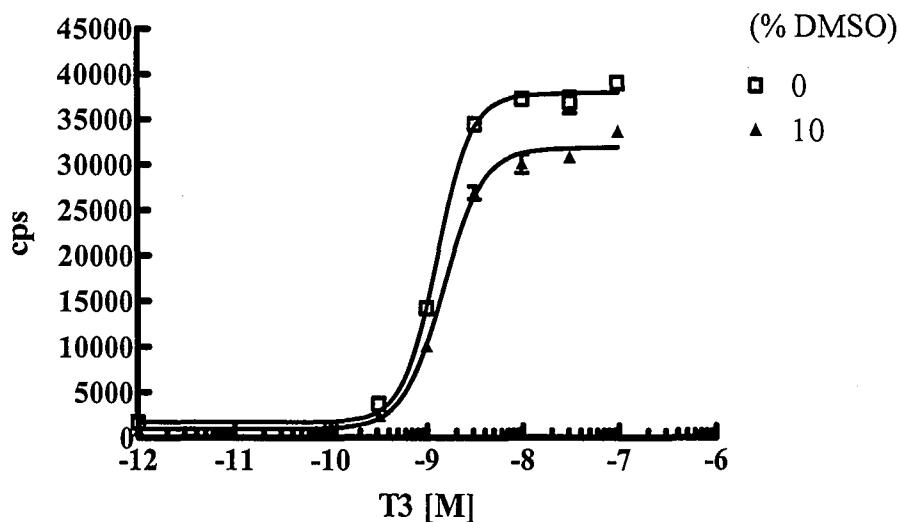
Fig. 8. Logistic concentration curves of T3 demonstrating DMSO tolerability in the biochemical assay.

3.2. Biochemical Evaluation of Thyroid Hormone Ligands in the Presence of Co-Regulators

Using these optimized conditions for the biochemical assay, the thyroid hormone

ligands T3, Triac, rT3, T4 and Tetrac were evaluated in logistic concentration curves

using TRα and TRβ proteins in the presence of co-activator peptide SRC1-2 and co-

repressor peptide NCoR. The concentration curves of T3, T4 and rT3 in the presence of

the co-activator SRC1-2 are demonstrated in **Fig. 9A, 9B** and **9C** respectively. The EC_{50}

values for these ligands are illustrated in **Table 2**. All of the ligands, with the exception

of Tetrac, appeared slightly more potent at TRα than TRβ. The order of potency of these

compounds for TRα from most potent to least was: Triac=T3>T4>Tetrac>rT3.

Interestingly, the order of potency of these compounds for TRβ, from most potent to least

was: Triac=T3>Tetrac>T4>rT3.
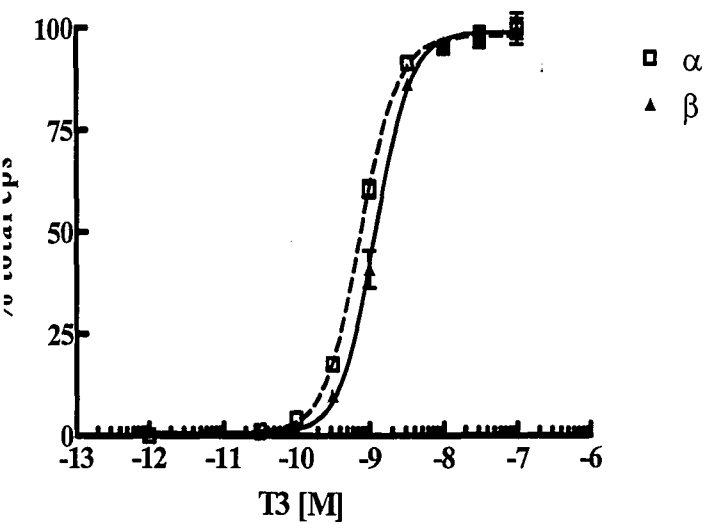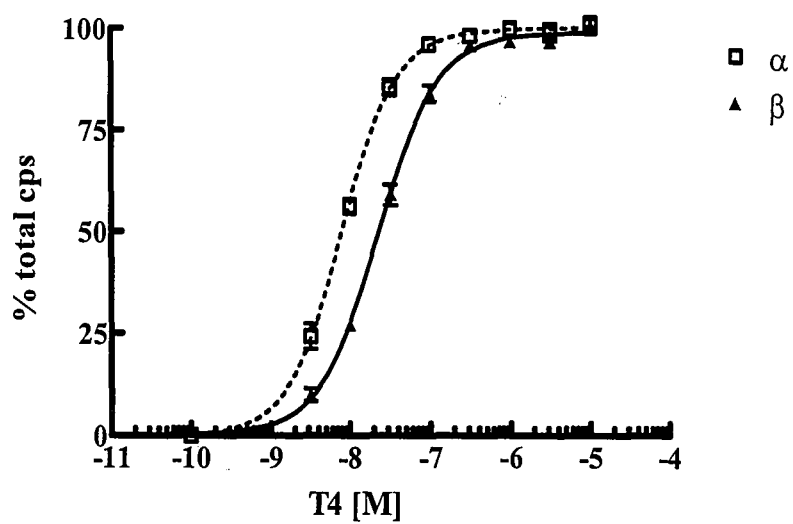
Fig. 9. Logistic concentration curves of thyroid hormones T3 (Panel A), T4 (Panel B) and rT3 (Panel C) in the biochemical assay, using TRα and TRβ in the presence of 10 nM SRC1-2 co-activator peptide.

Table 2. Potency and selectivity of thyroid hormone receptor ligands in a biochemical assay using either TRα or TRβ protein in the presence of SRC1-2 co-activator peptide.

Ligand	TRα EC$_{50}$ (nM $\pm$ SEM)	TRβ EC$_{50}$ (nM $\pm$ SEM)	Selectivity (TRβ/ TRα)
T3	0.77 $\pm$ 0.06	1.2 $\pm$ 0.10	1.6
Triac	0.74 $\pm$ 0.07	1.1 $\pm$ 0.14	1.5
rT3	57.1 $\pm$ 9.80	176.3 $\pm$ 25.4	3.1
T4	7.9 $\pm$ 0.80	22.7 $\pm$ 2.4	2.9
Tetrac	19.9 $\pm$ 3.70	12.5 $\pm$ 2.1	0.63

Graphical depiction of ligand logistic concentration response curves (Fig. 9; Panel A-C).
Data presented in the table were generated in a minimum of 2 separate assays done on different days. Each test concentration was run in duplicate.
EC$_{50}$ values represent half the maximal effective concentration of ligand.

These five ligands were then evaluated in logistic concentration curves using TRα and TRβ proteins in the presence of the co-repressor peptide NCoR. The concentration curves of T3, T4 and rT3 are demonstrated in **Fig. 10A, 10B** and **10C**, respectively. The EC$_{50}$ values for all of these ligands are illustrated in **Table 3**. The order of potency of these ligands for TRα from most potent to least was: T3>Triac>T4>Tetrac>rT3. The order of potency of these ligands for TRβ, from most potent to least was: T3=Triac> Tetrac>T4>rT3. Consistent with the SRC1-2 results, there is again a change in rank order potency of T4 and Tetrac between TRα than TRβ. The rank order of potencies for these compounds using both SRC1-2 and NCoR is similar to previously reported values [17]. However, in this earlier report, Tetrac was not included in the studies.
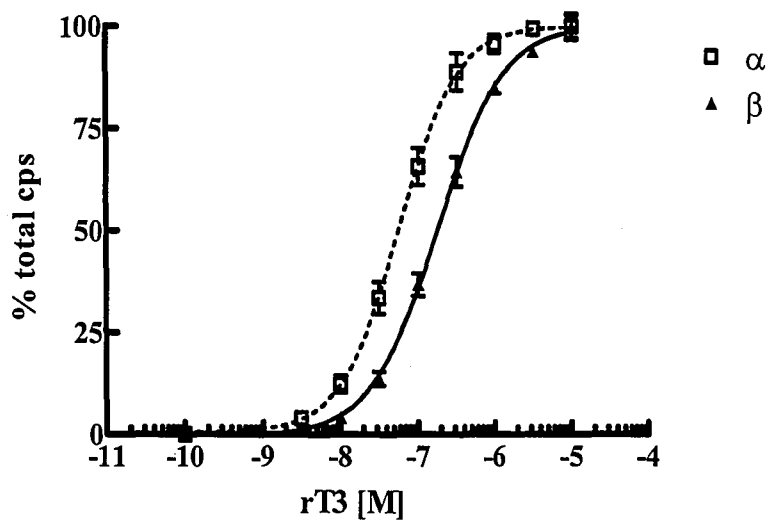
24
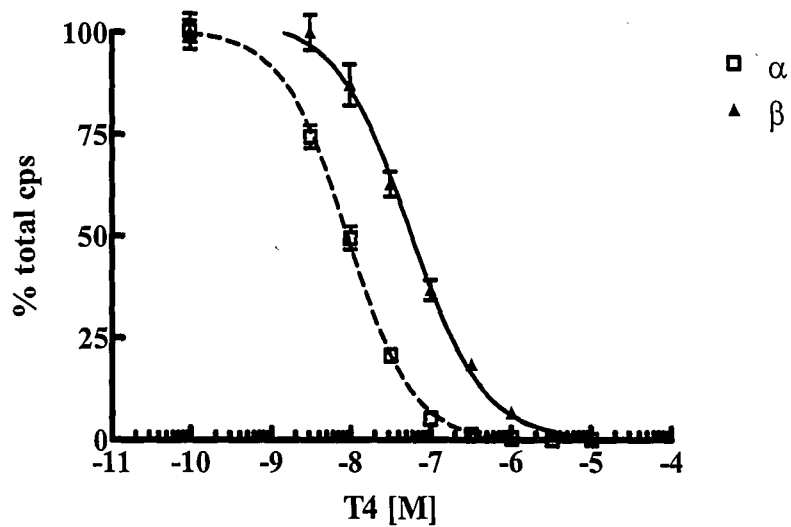
Fig. 10. Logistic concentration curves of thyroid hormones T3 (Panel A), T4 (Panel B) and rT3 (Panel C) in the biochemical assay, using TRα and TRβ in the presence of 10 nM NCoR co-repressor peptide.

Table 3. Potency and selectivity of thyroid hormone receptor ligands in a biochemical assay using either TRα or TRβ protein in the presence of NCoR co-repressor peptide.

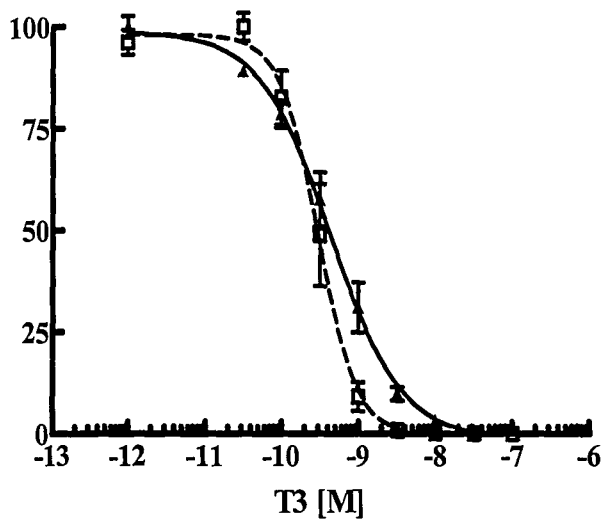| Ligand	TRα EC$_{50}$ (nM $\pm$ SEM)	TRβ EC$_{50}$ (nM $\pm$ SEM)	Selectivity (TRβ/ TRα)
T3	0.31 $\pm$ 0.07	0.44 $\pm$ 0.11	1.4
Triac	0.63 $\pm$ 0.11	0.58 $\pm$ 0.13	0.92
rT3	103.9 $\pm$ 19.2	423.5 $\pm$ 169	4.1
T4	9.2 $\pm$ 1.1	54.9 $\pm$ 11.5	6.0
Tetrac	19.5 $\pm$ 4.5	17.4 $\pm$ 5.1	0.90

Graphical depiction of logistic concentration response curves (Fig. 10; Panel A-C).
Data presented in the table were generated in a minimum of 2 separate assays done on different days. Each test concentration was run in duplicate.
EC$_{50}$ values represent half the maximal effective concentration of ligand.

3.3. Evaluation of Thyroid Hormone Ligands in Cell-Based Assay

In order to compare the activity of these compounds in the biochemical assay and a cell-based assay, they were evaluated in a One-Hybrid cell-based platform. It was first necessary to determine the concentration of DMSO tolerated in the cell-based assay. Logistic concentration curves of T3 were tested at varying concentrations of DMSO as seen in **Fig. 11**. It was apparent that concentrations greater than 1% DMSO decreased the overall cps signal, most likely due to cellular toxicity.
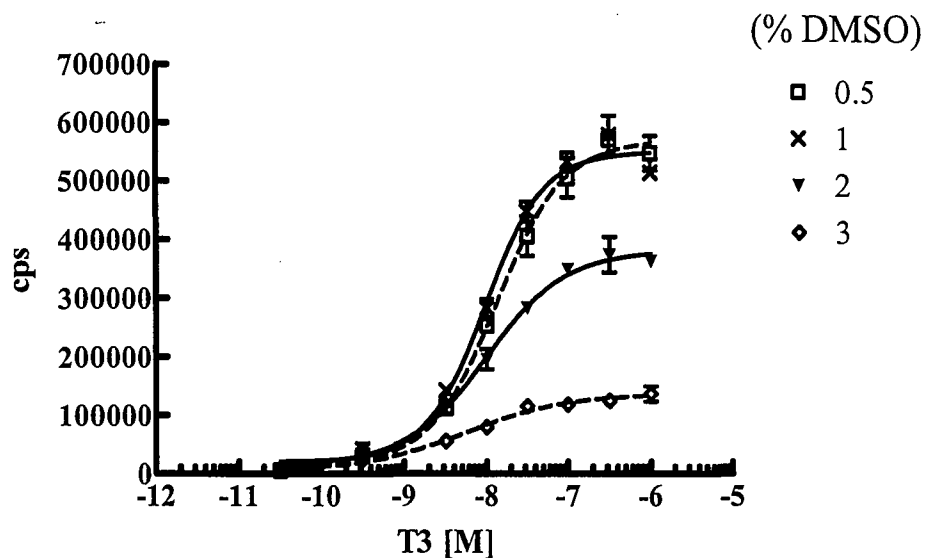
Fig. 11. Logistic concentration response curves of T3 demonstrating DMSO tolerability in the cell-based assay.

After this was determined, logistic concentration curves for T3, T4 and rT3 were tested as

seen in **Fig. 12A, 12B** and **12C**, respectively. The EC_{50} values for all of these ligands are

illustrated in **Table 4**. These ligands appeared less potent overall in the cell-based assay

than in the biochemical assay. However, the rank order of potency of these ligands were

the same for both TRα and TRβ in the cell-based assay. Most potent to least potent

being T3>Triac>T4>Tetrac>rT3. It should be noted that the exact EC_{50} values for rT3

are not reported as the dose response curves never reached a plateau at the higher

concentrations. This is most likely due to lack of complete solubility of the compound

because of the limiting DMSO concentration that is tolerated in this assay.

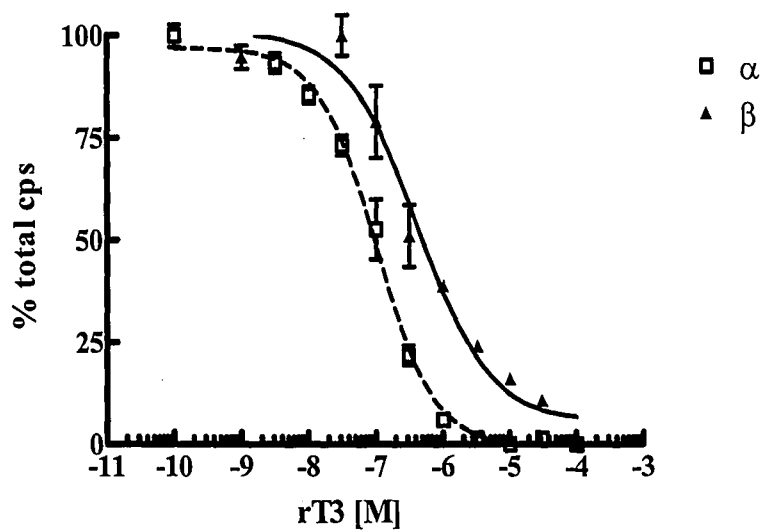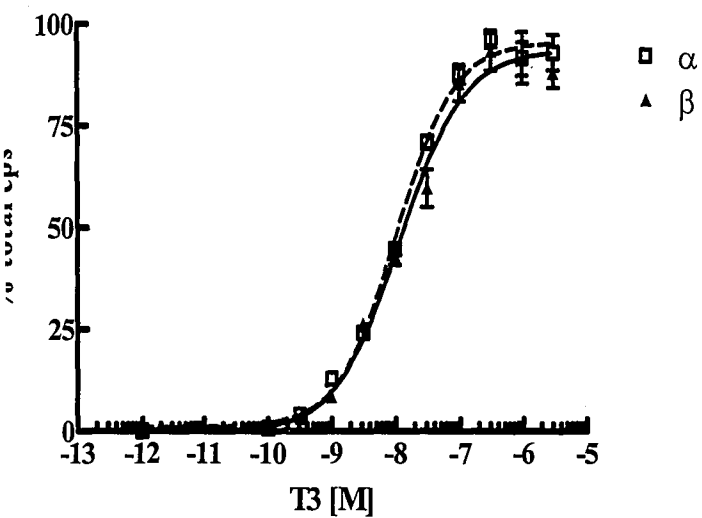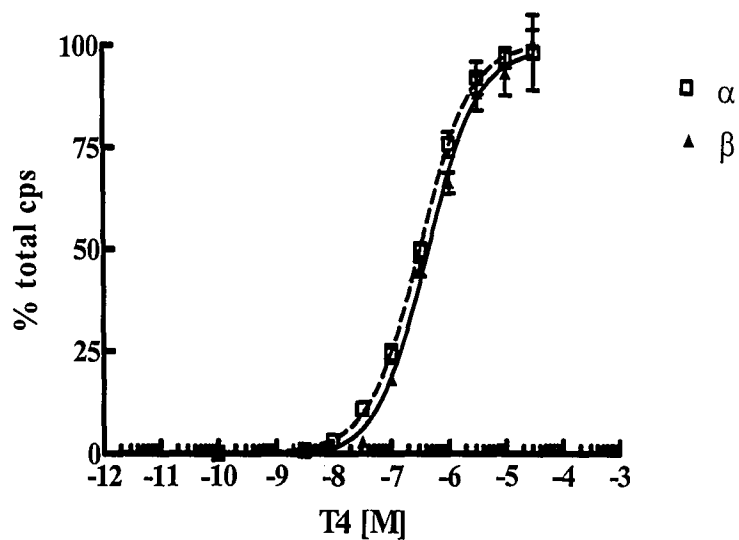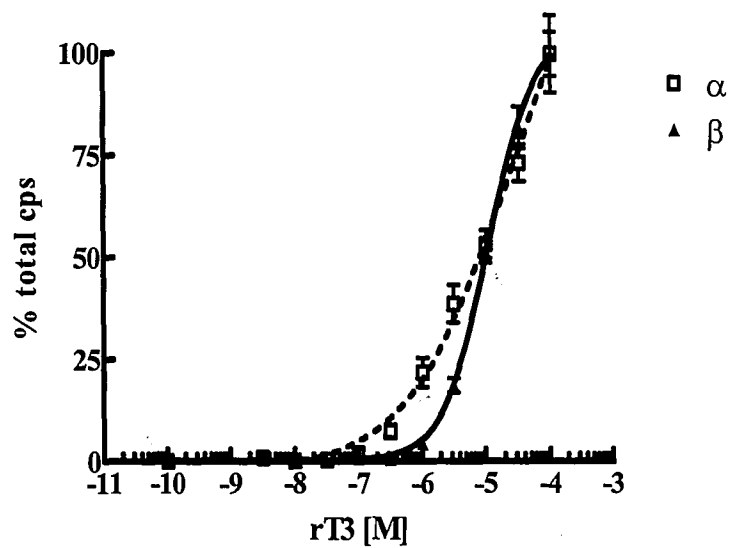**Fig. 12.** Logistic concentration curves of thyroid hormones T3 (Panel A), T4 (Panel B) and rT3 (Panel C), using TRα and TRβ in the cell-based assay.

Table 4. Potency and selectivity of thyroid hormone ligands in a cell-based assay using either the TRα or TRβ protein.

Ligand	TRα EC$_{50}$ (nM $\pm$ SEM)	TRβ EC$_{50}$ (nM $\pm$ SEM)	Selectivity (TRβ/ TRα)
T3	10.6 $\pm$ 2.0	11.8 $\pm$ 2.9	1.1
Triac	22.5 $\pm$ 5.6	12.1 $\pm$ 4.2	0.54
rT3	ND	ND	ND
T4	417 $\pm$ 81	317 $\pm$ 55	0.76
Tetrac	824 $\pm$ 176	493 $\pm$ 90	0.60

Graphical depiction of ligand logistic concentration curves (Fig. 12; Panel A-C).
Data presented in the table were generated in a minimum of 2 separate assays done on different days. Each test concentration was run in triplicate.
EC$_{50}$ values represent half the maximal effective concentration of ligand.
ND, not determined due to non-logistic concentration response

3.4. Comparison of Thyroid Hormone Ligand Affinities in the Biochemical and Cell-Based Assays

To demonstrate a direct comparison of the activities of these thyroid hormone ligands in the biochemical and the cell-based assays, logistic concentration curves of T3 at both TRα and TRβ, are illustrated in **Fig. 13A** and **13B**, respectively. It is apparent that the potency of T3 is significantly greater in the biochemical assay than in the cell-based assay. The EC$_{50}$ values for all of the ligands in both assays as well as the fold difference between the two values are reported in **Tables 5A** and **5B**. The EC$_{50}$ values for all of the ligands were more potent in the biochemical assay than in the cell-based

assay for both TRα and TRβ. However the fold differences when using the TRα protein

are greater between the assay platforms than when using the TRβ protein.

A
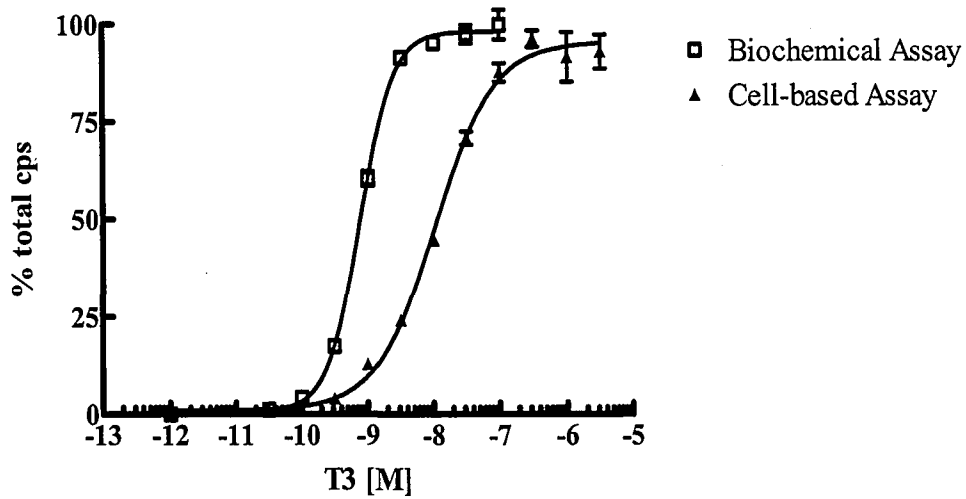
B

Fig. 13. Logistic concentration curves of thyroid hormone T3 at TRα (Panel A) and TRβ (Panel B)in the biochemical versus cell-based assays.

Table 5A. Comparison of EC50 values of thyroid hormone ligands between the cell-based and biochemical assays using TRα protein.

Ligand	Cell-based Bioassay EC$_{50}$ (nM ± SEM)	TRα-SRC1-2 Biochemical Bioassay EC$_{50}$ (nM ± SEM) [Fold Difference]	TRα-NCoR Biochemical Bioassay EC$_{50}$ (nM ± SEM) [Fold Difference]
T3	10.6 ± 2.0	0.77 ± 0.06 [13.8]	0.31 ± 0.07 [34.2]
Triac	22.5 ± 5.6	0.74 ± 0.07 [30.4]	0.63 ± 0.11 [35.7]
rT3	ND	57.1 ± 9.80 [ND]	103.9 ± 19.2 [ND]
T4	417 ± 81	7.9 ± 0.80 [52.8]	9.2 ± 1.1 [45.3]
Tetrac	824 ± 176	19.9 ± 3.70 [41.4]	19.5 ± 4.5 [42.3]

EC$_{50}$ values represent half the maximal effective concentration of ligand.
Fold difference is determined by dividing the less potent of the two EC$_{50}$ values of a given ligand generated between assays, by the more potent EC$_{50}$ value.
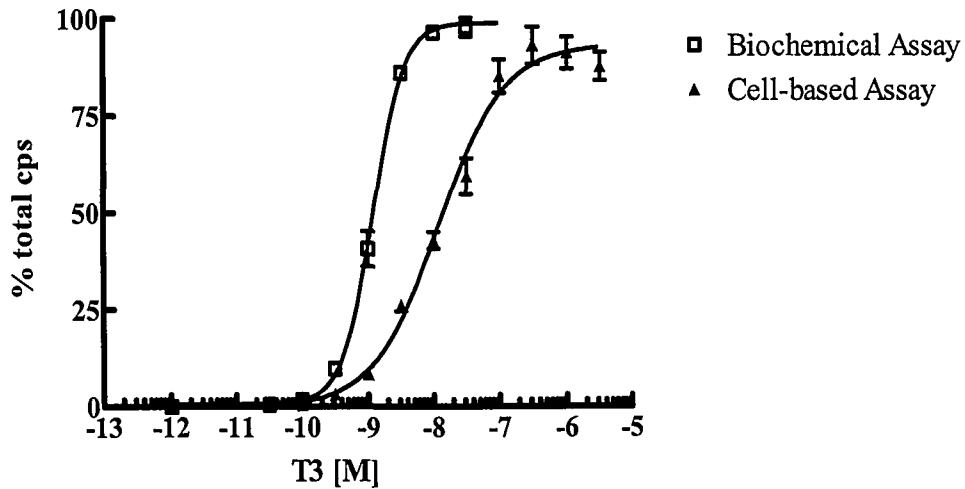ND, not determined due to non-logistic concentration response.

Table 5B. Comparison of EC50 values of thyroid hormone ligands between the cell-based and biochemical assays using TRβ protein.

Ligand	Cell-based Bioassay EC$_{50}$ (nM ± SEM)	TRβ-SRC1-2 Biochemical Bioassay EC$_{50}$ (nM ± SEM) [Fold Difference]	TRβ-NCoR Biochemical Bioassay EC$_{50}$ (nM ± SEM) [Fold Difference]
T3	11.8 ± 2.9	1.2 ± 0.10 [9.8]	0.44 ± 0.11 [26.8]
Triac	12.1 ± 4.2	1.1 ± 0.14 [11]	0.58 ± 0.13 [20.9]
rT3	ND	176.3 ± 25.4 [ND]	423.5 ± 169 [ND]
T4	317 ± 55	22.7 ± 2.4 [14]	54.9 ± 11.5 [5.8]
Tetrac	493 ± 90	12.5 ± 2.1 [39.4]	17.4 ± 5.1 [28.3]

EC$_{50}$ values represent half the maximal effective concentration of ligand.
Fold difference is determined by dividing the less potent of the two EC$_{50}$ values of a given ligand generated between assays, by the more potent EC$_{50}$ value.
ND, not determined due to non-logistic concentration response.

4. Discussion

Thyroid hormone receptors have been reported to affect numerous critical physiological processes and are activated or repressed by the binding of thyroid hormone. [6]. The two receptor subtypes of TR, TRα and TRβ, are of scientific interest as they have been implicated in a variety of disorders [11]. However, due to the lack of TR specific ligands, full characterization of these receptors, their specificity of function, and their specific mechanisms of action are largely unknown. Therefore, development of bioassays that can be used to identify new TR-selective ligands are essential in order to further elucidate the role of the TR subtypes in physiology. Thus, a biochemical assay was developed for the purposes of compound library screening and for the assessment of co-regulator interactions. In addition, a cell-based assay was developed that can be used in conjunction with the biochemical assay to more accurately predict endogenous and exogenous ligand specificity and receptor interaction. Following the optimization and development of these two bioassays, 5 known TR ligands were evaluated in parallel in these assays to characterize their potency and selective interaction with the TR subtypes.

The biochemical assay was developed using the AlphaScreen technology. The AlphaScreen platform was selected because it is suitable for high-throughput screening and amenable to studying co-regulator interactions. Assay optimization using both receptor subtypes was completed and the optimal protein concentrations, kinetic parameters and ligand carrier (tolerability) were determined. Because most compounds are soluble in DMSO, and this assay will be primarily used for compound screening, DMSO was chosen as the ligand dissolution carrier. The optimal protein concentration for both the TRα and TRβ-LBD was 5 nM at a 2 h (25°C) incubation time. It was

determined that this assay could tolerate up to a 10% final DMSO concentration without compromising the maximum signal or affinity value achieved for T3 at either receptor subtype. The fold difference between the maximum signal and the background was typically greater than 40-fold in this assay. Similar experiments were conducted in order to optimize the cell-based assay. In the cell-based assay, it was determined that the optimal plating density of cells in the assay was 10,000 cells per well. The assay was performed at 37°C, 5% CO_2, and allowed to incubate overnight. The maximum percentage of DMSO tolerated in the cell-based assay was 1% final concentration, which consistently allowed for approximately 20-fold difference between maximum and background signal for T3 at both receptor subtypes.

After completion of assay optimization, the 5 TR ligands were evaluated in the biochemical assay for TRα or TRβ in the presence of either the SRC1-2 co-activator or NCoR co-repressor peptide fragments. In the presence of SRC1-2, the rank order of potency between subtypes was different due to the reduced potency of T4 in the TRβ assay. The rank order of potency was T3=Triac> T4>Tetrac>rT3 in the TRα assay, whereas it was T3=Triac>Tetrac>T4>rT3 in the TRβ assay (Table 2). The T3 and Triac ligands demonstrated similar or equal potencies at both receptor subtypes, however the T4 ligand showed a 3-fold higher potency for TRα when compared to the potency noted for this ligand in the TRβ assay. The least potent ligand at both subtypes was the rT3 ligand, which, due to deiodinization at the tyrosyl ring, is essentially an inactive form of T4 known to have lower affinity for nuclear receptors [8]. Although this rT3 ligand showed similar rank order placement regardless of which receptor subtype was present, it did demonstrate a 3-fold increased selectivity for the TRα subtype. These data suggest

that based on the ligand structure, modest selectivity for a specific receptor subtype can be achieved in the presence of the SRC1-2 peptide. In the presence of the NCoR co-repressor peptide, the rank order of the ligands between subtypes was similar to the rank order affinities noted in the presence of the SRC1-2 peptide. The rank order of potency was T3>Triac> T4>Tetrac>rT3 in the TRα assay, whereas it was T3=Triac>Tetrac>T4>rT3 in the TRβ assay **(Table 3).** The T3 ligand demonstrated the highest potency overall when in the presence of NCoR peptide. Additionally, both the T4 and rT3 ligands maintained their selectivity for TRα that was noted in the presence of the SRC1-2 peptide. The rT3 ligand showed the lowest potency overall regardless of receptor subtype or co-regulator used.

In comparing the ligand potency differences in the presence of the two co-regulators, T3 and Triac demonstrated the highest potency in the presence of NCoR regardless of receptor subtype. It should be noted that in the presence of NCoR, the potency for the ligand is dependent on the ability of these ligands to dissociate the co-repressor peptide. Conversely, in the presence of SRC1-2, the ligand potency is dependent on the ability of the ligand/ receptor complex to recruit the co-activator peptide [17]. The dissociation or recruitment of the co-regulator is directly influenced by the conformation of the receptor in the presence of the ligand. Moreover, the co-regulator interaction with the receptor for the ligand/receptor complex can influence the affinity of the ligand, as well as determine the functional consequence of the binding interaction[18]. Interestingly, it was observed that the potency of Tetrac was not affected regardless of the co-regulator present or the receptor subtype. Tetrac is a deamination product of T4 and has been shown to block thyroid hormone binding, but has no agonist

activity at the hormone receptor site [19]. These results indicate that the binding of Tetrac to the ligand binding domains of TRα and TRβ induces a conformation which is equally conducive to co-repressor dissociation and co-activator recruitment. In addition, these data may reflect the involvement of the non-agonist characteristics of Tetrac that have been reported [20]. It is imperative to use caution when interpreting the data derived in the biochemical assay. The biochemical assay is a very simplistic bioassay that is designed to allow for screening large compound libraries. The co-regulators used in this assay are only peptide fragments of the full-length co-regulators that are expressed endogenously. The ratio of the receptor to the co-regulator is critical and will determine functional consequence of the interaction. In the biochemical assay the receptor and co-regulator content is kept constant, therefore, this ratio may not directly translate to the receptor/co-regulator ratios found in various cell lines or tissues. Also, only 2 of the known TR co-regulators were evaluated in the biochemical assay. Therefore, the higher affinity noted for T3 in the presence of NCoR, the selectivity of rT3 and T4 for TRα, and the lack of affinity differences with Tetrac regardless of co-regulator or receptor subtype may not be representative when evaluating these ligands in cell-based or tissue preparations. Thus, the importance of evaluating these ligands in a cell line that can express the ligand binding domain and all full-length TR co-regulators is critically important to assure the findings will translate endogenously. Therefore, a cell-based assay was developed using the COS-7 cells. These cells were selected because they are easily transfectable with the TR subtypes. Overall, the potencies of the ligands tested were reduced when compared to the data generated from the biochemical assay. This shift may be due to the fact that the full complement of co-regulators are present, and the

change in potency may be a result of the occurrence of some repression counteracting the activation of the receptors. More importantly, there was no selectivity noted between the subtypes for either T4 or rT3 that was previously demonstrated in the biochemical assay. In fact, for the rT3 ligand, the potency shift was significant enough that the concentrations necessary to produce a complete concentration curve were too high to maintain the ligand in solution at the maximum percentage of DMSO tolerated in this assay. Therefore, these concentration-response curves did not reach plateau, prohibiting the estimation of an accurate EC_{50} value.

Taken together, the major pharmacological difference noted between the biochemical and the cell-based assay was the lack of selectivity translation in the cell-based assay that was shown by T4 and rT3 for TRα in the biochemical assay. Also, an overall reduced potency for the ligands was noted in the cell-based assay when compared with the biochemical assay. However, as the potencies of the ligands were reduced in the cell-based assay, the rank order of the ligands remained similar. Ultimately, in trying to understand or establish a pharmacological profile of these ligands or any experimental compound, the cell-based assay would be most relevant. The cell-based assay provides the endogenous make-up of the co-regulators that are critically involved in receptor-mediated function and are present in various varieties and densities in different tissues. Therefore, the potency as well as the selectivity for any given compound will be tissue context-dependent. However, this fact should not minimize the importance of the biochemical assay. The development of such an assay allows for accelerated screening of a large number of compounds as well as an overall rank ordering of compound affinity for a given receptor subtype. In addition, the biochemical assay may provide important

information for chemical structure modification that may lead to compounds that are receptor-selective in both biochemical and cell-based assays.

5. Conclusion

From this study, it was made apparent that the affinity of thyroid hormone ligands and their derivatives, for TRα and TRβ is dependent on not only the assay platform, but also the co-regulator present. However, the rank order of potencies of the thyroid hormone ligands, regardless of which co-regulator was present, remained similar. Interestingly, there was a change in rank order of potencies between the TRα and TRβ subtypes for the T4 and Tetrac ligands. This could provide further insight into the binding of ligands to TR receptor subtypes. Also, it was noted that the ligands T4 and rT3 demonstrated selectivity in the biochemical assay but not in the cell-based assay. This further emphasizes the distinction between assay formats, but more specifically a distinction between the interaction of thyroid hormone ligands with TRα and TRβ in a synthetic environment as opposed to one that is more representative of a naturally occurring environment.

In summary, a biochemical and cell-based assay was developed and optimized in order to characterize known TR ligands at both TRα and TRβ subtypes and to further understand their interactions. Our findings provide an initial assessment of these ligands for the TR subtypes as well as the influence of 2 co-regulators on the ligand-receptor interaction. This information will be used to further explore the receptor subtype differences and undertake a medicinal chemistry effort to develop small molecule

agonists and antagonists that will discriminate between the 2 receptor subtypes and allow

for further understanding of their role in the physiology of TR biology.

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