Analysis of the roles of mutations in thyroid hormone receptor-β by a bacterial biosensor system

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Abstract
Mutations in thyroid hormone receptors (TRs) often lead to metabolic and developmental disorders, but patients with these mutations are difficult to treat with existing thyromimetic drugs. In this study, we analyzed six clinically observed mutations in the ligand-binding domain of the human TRβ using an engineered bacterial hormone biosensor. Six agonist compounds, including triiodothyronine (T3), thyroxine (T4), 3,5,3'-triiodothyroacetic acid (Triac), GC-1, KB-141, and CO-23, and the antagonist NH-3 were examined for their ability to bind to each of the TRβ mutants. The results indicate that some mutations lead to the loss of ability to bind to native ligands, ranging from several fold to several hundred fold, while other mutations completely abolish the ability to bind to any ligand. Notably, the effect of each ligand on each TRβ mutant in this bacterial system is highly dependent on both the mutation and the ligand; some ligands were bound well by a wide variety of mutants, while other ligands lost their affinity for all but the WT receptor. This study demonstrates the ability of our bacterial system to differentiate agonist compounds from antagonist compounds and shows that one of the TRβ mutations leads to an unexpected increase in antagonist ability relative to other mutations. These results indicate that this bacterial sensor can be used to rapidly determine ligand-binding ability and character for clinically relevant TRβ mutants.

Key Words
- thyroid hormone receptor
- ligand-binding assay
- hormone receptor mutations
- engineered biosensor

Introduction
Thyroid hormone receptors (TRs) belong to the nuclear hormone receptor (NHR) superfamily, which includes a wide variety of transcriptional regulators that respond to various small-molecule hormones. TRs specifically regulate those genes that respond to various levels of native thyroid hormones. Similar to other NHRs, TRs can be structurally divided into several domains: two conserved activation function domains (AF1 and AF2) and a DNA-binding domain (DBD) connected to the ligand-binding domain (LBD) through a hinge domain (Zhang & Lazar 2000). In humans, there are two TR subtypes, α and β, expressed by two different genes located on chromosomes 17 and 3 respectively (Cheng 2000, Zhang & Lazar 2000). Alternate RNA splicing gives rise to several isoforms for each subtype, some of which cannot respond to thyroid hormone ligands. Of the TRα isoforms, only TRα1 exhibits the ability to bind to the native thyroid hormone triiodothyronine (T3), while all the three TRβ isoforms have T3-binding ability. The expression levels of the various TRs within a given cell depend on both tissue type and developmental stage for a given animal (Cheng 2000).
TRs play a critical role in mammalian metabolism and development through the regulation of gene transcription. A zinc-binding domain in the TR DBD binds to a double-stranded DNA at specific sequences, forming a homodimer or heterodimer with retinoid X receptor (RXR). In the absence of thyroid hormones, TR/RXR heterodimers bind to co-repressors, such as the nuclear receptor co-repressor (N-CoR), or the silencing mediator of retinoid and thyroid receptors (SMRT). These complexes contain histone deacetylases, which create a closed chromatin conformation leading to transcriptional repression (Fondell 2013, Pascual & Aranda 2013). In the presence of thyroid hormones, the conformation of TRs changes to destabilize co-repressor binding and favor the recruitment of transcriptional co-activators, leading to gene transcription. Depending on the co-activators and co-repressors present in the cell, TRs can sometimes repress gene expression in the presence of a ligand or activate gene expression without thyroid hormones (Pascual & Aranda 2013, Sirakov et al. 2013).

Resistance to thyroid hormone (RTH) is an inherited syndrome that reduces tissue sensitivity to thyroid hormones (Cheng 2005). RTH is characterized in vivo by an increased level of free thyroid hormone or thyroid-stimulating hormone (TSH) in the serum, while TR-dependent gene expression is not increased. Because of the importance of TRs in metabolism and development, RTH leads to many clinical symptoms, such as goiter, weight loss, hair loss, short stature, decreased IQ, and dyslexia. Increasing evidence suggests that RTH is primarily related to TR mutations, although mutations in co-activators, co-repressors, and other proteins related to thyroid metabolism (e.g., selenoproteins) can also lead to these disorders (Refetoff & Dumitrescu 2007).

The high homology of TRs across animal species has allowed RTH models to be developed using engineered mice, zebrafish, and tadpoles (Cheng 2005, Porazzi et al. 2009, Grimaldi et al. 2013). Unfortunately, these animal models do not lend themselves to high- or medium-throughput drug screening. For this reason, an inexpensive, microtiter assay for RTH therapeutics is highly desirable, especially for preclinical drug discovery and development.

In our previous work, we created several hormone biosensors through gene fusions of animal NHR LBDs to a convenient reporter protein scaffold. The resulting fusion proteins produce hormone-dependent growth phenotypes when expressed in modified Escherichia coli cells (Skretas & Wood 2005a, b, Li et al. 2011). In particular, the binding of an appropriate ligand to the fused LBD activates a thymidylate synthase (TS) reporter enzyme, which allows the E. coli strain D1210ΔthyA expressing this protein to grow in media lacking thymine. This sensor is capable of differentiating agonist compounds from antagonist compounds and has shown utility in the identification and characterization of a number of compounds that bind to human and animal estrogen receptors (ERs; Gawrys et al. 2009, Gierach et al. 2013). More recently, this strategy has been validated for subtype-selective human TR ligands using a small library of known selective ligands (Gierach et al. 2012).

In this study, we introduced six clinically relevant mutations into our human TRβ biosensor (Fig. 1) and examined the resulting effects on the ability to bind to recognized native and synthetic TR ligands. These include the native agonists T3 and thyroxine (T4), as well as the synthetic agonists 3,5,3′-triiodothyroacetic acid (Triac), GC-1, and KB-141, and the synthetic antagonist NH-3. Our results indicate that the effects of these TRβ mutations on ligand-binding are not equal, and that the binding of both agonists and antagonists is highly dependent on each specific mutation. Some mutations lead to partial loss of ability to bind to some ligands (EC50 values increase several to several hundred times), while other mutants completely lost binding ability for all ligands. Surprisingly, one of the mutants has shown a significant increase in NH-3 growth inhibition relative to the WT receptor in the presence of T3, suggesting that this mutation may disrupt T3 interactions with the binding pocket, while leaving those for NH-3 more intact.

Materials and methods
Reagents
The ligands Triac (95%), T3 (sodium salt hydrate, 95%), 17-estradiol (E2), and T4 were purchased from Sigma. The ligands GC-1 (3,5-dimethyl-4-(4′-hydroxy-3′-isopropylbenzyl) phenoxy acetic acid) and KB-141 (3,5-dichloro-4-(4′-hydroxy-3′-isopropylphenoxy) phenyl acetic acid) were a gift from Dr Gary Grover (University of Medicine and Dentistry of New Jersey, Newark, NJ, USA). The ligands NH-3 (2-(4-((4-hydroxy-3-(1-methylethyl)-5-(2-(4-nitrophenyl)ethynyl)phenyl)methyl)-3,5-dimethylphenoxy) acetic acid) and CO-23 (5-((4-(4-hydroxy-3-propan-2-ylphenoxy))-3, 5-diiodophenyl)methyl)imidazolidine-2, 4-dione) were a gift from Tom Scanlan (Oregon Health & Science University, Portland, OR, USA). All other chemicals used for microbial cell culture were purchased from Fisher Scientific (Pittsburgh, PA, USA).
Construction of mutant sensors

The mutant sensors are all based on our previously reported human TRβ sensor fusion protein. Six mutations, R243W, N331D, R338W, L346F, Δ430M, and P453H, were introduced into the human TRβ sensor LBD through overlap extension PCR using two pairs of primers annealing to the intein backbone and the mutation sites respectively (Supplementary Table S1, see section on supplementary data given at the end of this article). Overlap extension PCR products were then digested with AgeI and XhoI and ligated into pMIT:TRβ (Skretas & Wood 2005a) to replace the WT sensor and generate the mutant sensors. All the mutant constructs were confirmed by sequencing.

Determination of bacterial sensor growth phenotypes

The constructed mutant sensor plasmids were transformed into the TS (TYMS)-deficient E. coli strain D1210ΔthyA::KanR (FΔ (gpt-proA) 62 leu B6 sup E44 ara-14 galK2 lacY1Δ (mcr-c-mrr) rpsL20 (StrR) xyl-5 mtl-1 recA13 lacY1) and cultured on Luria-Bertani (LB) agar plates supplemented with 50 μg/ml thymine. The determination of phenotypes was carried out in the presence of various ligands consistently with our previously reported work (Gierach et al. 2012). The transformed cells were then cultured in 3 ml LB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml thymine and incubated at 37 °C to an OD600 value of 1.2. These seed cultures were then diluted 1:200 in liquid thymine-free medium (-THY) (per liter: 10 ml of 20% glucose; 200 ml of 5% casamino acids; 10 ml of 0.1 M MgCl2, pH 7.0) supplemented with 100 μg/ml ampicillin. For the antagonist assay, seed cultures were diluted in liquid thymine-free medium supplemented with 10 μM T3. For growth assays, 200 μl of the diluted cells were transferred into each well of a 96-well microtiter plate, and each well was supplemented with 2 μl of ligands dissolved in DMSO. Importantly, the concentration of ligand in DMSO was adjusted such that the total concentration of DMSO in each well was the same and only the concentration of ligand varied. The 96-well plates were then incubated at 34 °C, 150 r.p.m. agitation, and 80% humidity for 22 h. Growth phenotypes were then measured by optical absorbance at a wavelength of 600 nm (OD600) using a Biotek Synergy 2 plate reader. All the assays were carried out in triplicate, and each was additionally repeated three times on three different days. The reported growth signal is the difference between the OD600 value in the presence of a ligand and that in the presence of the pure DMSO control for each mutant. The inhibition efficiency for the NH-3 antagonist is defined as the ratio of the growth signals in the presence of both NH-3 and T3 to the growth signals in the presence of T3 alone.

Statistical analysis

Growth responses in each case are based on the average of three independent experiments carried out on three different days, where each experiment was carried out in triplicate (a total of nine data points). Error bars in each figure represent a single s.d. from the mean for all the data points measured. The half-maximal effective concentration was determined by fitting the growth data to equation (1), where X is the ligand concentration, Y is the observed growth signal, and Bottom, Top, EC50, and Hillslope are the fitted parameters. The fitting was carried out using the software package OriginPro 8 (www.originlab.com):

\[ Y = \frac{\text{Top} - \text{Bottom}}{1 + 10^\left( \log EC50 - X \right \cdot \text{Hillslope} \right) + \text{Bottom}} \]

The statistical significance (P value) between the signals from the wide-type and mutant receptors was determined via Student’s t-test function (two-tailed, two-sample equal variance) in Microsoft Excel 2010.

Results

The LBD of TRβ contains twelve α-helixes and four β-strands. In this study, we examined six previously reported mutations, R243W (Pohlenz et al. 1996), N331D (Rivolta et al. 2009), R338W (Weiss et al. 1993), L346F (Rivolta et al. 2009), Δ430M (Collingwood et al. 1994), and P453H (Shuto et al. 1992), which are well distributed throughout the LBD sequence (Fig. 1 and Supplementary Figure S1, see section on supplementary data given at the end of this article). In particular, R243W is located at the end of helix 2 and therefore close to the N terminus of the LBD. The N331D and L346F mutations are located on a long loop between helix 6 and helix 7. The L346F mutation is located in the loop between helix 7 and helix 8, while the Δ430M and P453H mutations are located in helix 11 and helix 12 respectively (Fig. 1B and Supplementary Figure S1).

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Each mutation was introduced into the native TRβ LBD through overlap extension PCR using two pairs of primers for each mutation, and all clones were confirmed by sequencing prior to testing (Supplementary Table S1).

Agonist assay

Growth phenotypes of the mutant sensors The native TRβ sensor and the six mutant sensors were first exposed to a small library of TR agonists, which included T₃, T₄, Triac, GC-1, KB-141, and CO-23. Of these ligands, CO-23 has been reported to bind to TRα and TRβ with a similar affinity, but it appears to preferentially activate TRα in some assays (Kawakami et al. 2008, Grijota-Martínez et al. 2011). Phenotype tests were carried out similarly to our previous work, where the E. coli biosensor cells were grown in -THY liquid medium (see the Materials and methods section) with each ligand supplied at a final concentration of 10 μM using a DMSO vehicle. Growth was quantified by measuring absorbance at 600 nm (OD₆₀₀), where the background is based on a pure DMSO vehicle as a control. The responses of the native and mutant sensors to the tested ligands are shown in Fig. 2. All the reported agonists, including CO-23, generated a significant growth response in the WT TRβ biosensor.

The behaviors of the TRβ mutant sensors varied, and the mutants could be divided into three groups based on the responses. The first group contained the R243W and R338W mutants, which responded to all the tested agonists. However, the signals observed for this group were generally lower than those observed for the native sensor. Notable exceptions were the Triac and GC-1 signals, which were found to be similar or higher. The second group contained the N331D and ∆430M mutants, which did not respond to any of the agonists tested. The lack of response was characterized by no significant difference between the test ligand and vehicle-only background after 22 h of incubation (ΔOD₆₀₀ < 0.01). The third group contained the L346F and P453H mutants, which responded variably to some of the ligands. In particular,

![Figure 1](Image)

**Figure 1** Human TRβ bacterial sensor protein design with tested mutations on ribbon diagram structure. (A) Schematic representation of the human TRβ bacterial sensor protein. The sensor fusion protein contains four domains: MBP is a maltose-binding protein at the N terminus of the sensor protein, which is believed to normalize expression and increase the solubility of the fusion protein; hTRβ is the human TRβ LBD, which is the ligand recognition element of the sensor; IN and IC are the N- and C-terminal segments of a split intein, which is believed to stabilize the inserted TRβ LBD; and TS is a thymidylate synthase reporter protein, which generates a growth phenotype response when it is activated. The biosensor fusion is designed such that ligand-dependent conformational changes in the LBD are transferred through the intein domain to the TS domain, leading to the activation of the TS domain and generation of a readily detectable change in growth phenotype. The six mutations investigated in this study are shown below. (B) The structure of the human TRβ in complex with T₃ (protein data bank ID#: 3GWS) indicating the mutations examined in this study. The T₃ ligand is shown at the center in a colored ball-and-stick representation, where the side chains of the mutation locations (WT side chains are shown) are indicated by white ball-and-stick representations.

![Figure 2](Image)

**Figure 2** Growth phenotypes of the mutant and the native sensors. Mutant bacterial sensors were cultured in 96-well plates in -THY medium as described in the Materials and methods section. Each well was supplemented with the indicated TRβ agonists to a final concentration of 10 μM, with pure DMSO as a vehicle background control. Cell densities were read at 600 nm (OD₆₀₀) after incubation at 34 °C for 22 h. The signals shown above are the OD₆₀₀ of each well above DMSO background for each mutant (subtracted OD₆₀₀). Error bars indicate one s.e. from three replicate experiments. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-13-0108.
the L346F mutant responded strongly to Triac alone, while the P453H mutant responded to T3, Triac, and GC-1. The Triac signal was notably higher in the mutant sensors than in the native sensor for all the mutants that responded to Triac (R243W, R338W, L346F, and P453H). Each differential response was analyzed for statistical significance (Table 1), and in nearly all the cases, the differences in responses were found to be highly significant. The primary exception was the responses of the WT sensor and the L346F mutant to Triac, where the P value was 0.35. This low significance is an artifact of a similar binding affinity of both these receptors for this ligand.

**Active mutant sensor responses to agonists** For each ligand and mutant pair where a growth signal was observed, a dose–response curve was generated to evaluate the effect of each mutation on the apparent relative binding affinity for the ligand. The effects are reported in terms of the ligand’s EC50 (half-maximal effective concentration) value for each mutant LBD (Table 2). Importantly, the dose–response curves became saturated at the highest concentration (100 mM) for all the ligands in combination with the native LBD, but curves for some of the LBD mutants were unable to reach saturation at this concentration in combination with some ligands (Fig. 3). Unfortunately, solubility limitations of the test ligands in DMSO and -THY medium prevented testing at concentrations higher than 100 μM, which generated some uncertainty in some tests.

The effects of ligands on cell growth exhibited dependence on both the TRβ mutation and the identity of the ligands, whereby the binding ability of some ligands was affected by some mutations more than others (Fig. 3 and Table 2). For example, the EC50 values observed for the native T3 agonist to which the first mutant group was exposed (7.69×10⁻⁶ M for the R243W mutant and 8.76×10⁻⁶ M for the R338W mutant) indicated approximately seven times weaker binding ability compared with that for the native sensor (1.21×10⁻⁶ M). These changes are consistent with the previously reported values for similar mutations at the same position (R243Q) (Huber et al. 2003). In the third mutant group, decreases in binding affinity for T3 were much larger than those in the first mutant group. In particular, the P453H mutant exhibited a binding affinity for T3 that was about 50-fold lower than that exhibited by the native receptor (EC50 value for the P453H mutant was 5.80×10⁻⁵ M), while the L346F mutant exhibited no T3-binding affinity. The native

**Table 1** Calculated P values for the data shown in Fig. 2.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>R243W</th>
<th>R338W</th>
<th>N331D</th>
<th>Δ430M</th>
<th>L346F</th>
<th>P453H</th>
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<tr>
<td>T3</td>
<td>0.083</td>
<td>0.016</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.00054</td>
</tr>
<tr>
<td>T4</td>
<td>0.00022</td>
<td>0.00013</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Triac</td>
<td>0.0024</td>
<td>0.011</td>
<td>NA</td>
<td>NA</td>
<td>0.35</td>
<td>0.0066</td>
</tr>
<tr>
<td>GC-1</td>
<td>0.000052</td>
<td>0.00011</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.00075</td>
</tr>
<tr>
<td>KB-141</td>
<td>0.019</td>
<td>0.030</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CO-23</td>
<td>0.00017</td>
<td>0.000172</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, no activity.

**Table 2** Summary of the EC50 values of different ligands for wild-type and mutant sensors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensors</th>
<th>Agonist (M)</th>
<th>Antagonist (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T3</td>
<td>T4</td>
<td>Triac</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>1.21×10⁻⁶</td>
<td>8.31×10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>R243W</td>
<td>7.69×10⁻⁶a</td>
<td>6.94×10⁻²a</td>
</tr>
<tr>
<td></td>
<td>R338W</td>
<td>8.76×10⁻⁶a</td>
<td>2.94×10⁻²a</td>
</tr>
<tr>
<td>2</td>
<td>N331D</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Δ430M</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>L346F</td>
<td>NA</td>
<td>2.84×10⁻⁶a</td>
</tr>
<tr>
<td></td>
<td>P453H</td>
<td>5.80×10⁻⁵a</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, no activity.

*The dose–response curve does not reach saturation.
T₄ ligand, which is generally thought to be a storage form of T₃ in vivo, was also bound by the native receptor and the first mutant group, but in all cases it showed a significantly lower binding ability than T₃ (Table 2).

Triac, which is a strong thyroid hormone mimic, was able to bind to all the mutants in the first and third mutant groups and was the only ligand to be bound by the L346F mutant. Despite its unique binding ability, the EC₅₀ value of Triac for the L346F mutant was over 600 times higher than that for the native receptor (Table 2), and the dose–response curve failed to reach saturation. The Triac EC₅₀ values for the R338W and R243W mutants are 10-fold and 20-fold higher than the native sensor, respectively, and approximately 100-fold higher for the P453H mutant, indicating weaker binding for all of the mutants tested.

The responses of the native and mutant sensors to the agonist mimics GC-1 and KB-141 were different from those to T₃, T₄, and Triac. Surprisingly, the observed EC₅₀ values of GC-1 and KB-141 for the R243W and R338W mutant sensors indicated a stronger or similar binding ability compared with that for the native sensor. By contrast, T₃, T₄, and Triac exhibited a much weaker binding ability for these mutants (Fig. 3 and Table 2).

The synthetic TRα selective ligand, CO-23, was also tested. Although CO-23 binds with a similar affinity to both TR isoforms, it exhibits fivefold greater activation for TRα than for native TRβ (Ocasio & Scanlan 2006). In our single-dose assays, CO-23 showed significant binding to only the native TRβ LBD and the R243W mutant. The EC₅₀ value of CO-23 was only approximately threefold higher than that of T₃ for the native TRβ sensor, indicating a slightly weaker binding ability, but it exhibited a stronger binding ability than the TRβ selective agonists GC-1 and KB-141 (Table 2). The R243W mutation...
decreased the CO-23-binding ability by approximately sixfold compared with that of the native receptor (3.42 × 10⁻⁶ vs 1.91 × 10⁻⁵ M).

**Antagonist assay**

Several compounds have been reported as known or suspected TR antagonists, including bisphenol-A (Moriyama et al. 2002, Zoeller et al. 2005), 3,5-dibromo-4-(3',5'-diisopropyl-4'-hydroxyphenoxo)-benzoic acid (Baxter et al. 2002), GC-14 (Nguyen et al. 2002), NH-3 (Lim et al. 2002, Nguyen et al. 2002), and a rationally designed chimera combining the TR ligand GC-1 with the alkylamide appendage of the estrogen antagonist ICI 184 384 (Yoshihara et al. 2001). Of these, NH-3 is widely recognized and has been studied both *in vivo* and *in vitro* (Lim et al. 2002, Figueira et al. 2011). For these reasons, we evaluated NH-3 for its ability to antagonize the effects of T₃ on the native receptor, as well as on the R243W and R338W mutant receptors. Tests were carried out in -THY medium supplemented with 10 μM T₃ to allow intermediate growth of the sensor strain, while an ERβ (ESR2)-expressing strain would be somewhat stronger preference for NH-3 than for T₃. These data indicate that NH-3 is an antagonist for the native TRβ strain, and that the R243W mutant was inhibited to a level of ≈50% for equimolar T₃ and NH-3 (10 μM each), indicating close to equal binding affinity of each mutant for each ligand. However, the R243W mutant was inhibited to a level of ≈75% under these conditions, implying that this mutant exhibits a somewhat stronger preference for NH-3 than for T₃.

To quantify the effect of these mutations on NH-3-binding ability, the EC₅₀ values for these three sensors were calculated from the dose–response curves (Fig. 4B). Unlike the agonists, the fitted EC₅₀ curves for the mutant sensors were shifted to the left of the WT receptor (3.79 × 10⁻⁶ vs 1.09 × 10⁻⁵ M), while the value for the R338W mutant was close to that for the native receptor (9.64 × 10⁻⁶ vs 1.09 × 10⁻⁵ M).
Discussion

In this study, we employed an engineered bacterial sensor to study the role of RTH-associated mutations in the human TRβ LBD. Using our system, we examined the impacts of six mutations distributed throughout the TRβ LBD sequence on ligand-binding ability. The results indicate that our sensor system can distinguish between mutant and WT receptors and can provide quantitative estimates of the potential effects of different LBD mutations on ligand-binding ability. In particular, our results suggest that different mutations affect ligand-binding activities differently, whereby some mutations completely abolish the binding activity for any ligand, while others have varying ligand-specific effects. Finally, we found one mutation to increase the sensitivity of the LBD to the NH-3 agonist relative to T3, despite this mutant exhibiting a generally lower agonist-binding activity.

As with many nuclear receptors, the TRβ ligand-binding pocket lies at the core of its C-terminal LBD (Wagner et al. 1995). Structural observations of the TRβ LBD bound to T3 indicate that there are 13 residues that take part in ligand binding (Nascimento et al. 2006). Specific mutations at two of these residues, N331D and L346F, were examined in this study. Notably, N331D is the only difference between TRβ and TRx in the ligand-binding pocket and is thought to play an important role in ligand selectivity for these two TR subtypes (Wagner et al. 2001). The importance of these two residues in ligand recognition and binding may partially explain why these two mutations abolish the ability to bind to the native T3 agonist. Conversely, the Δ430M mutation is far from the binding pocket, but is located in middle of the structurally critical helix 11. Deletion of this residue would significantly affect the configuration of this helix, probably resulting in instability of the whole structure (Fig. 1B). Both the R243W and R338W mutations are outside of the binding pocket and are therefore likely to have more subtle effects on binding and stability. This would be consistent with their observed ability to bind to all the tested ligands in this study, with varying effects on the observed EC50 dose–response values. Interestingly, a mutation similar to R243W (R243Q) has been studied by crystallography and B-factor analysis (Huber et al. 2003). The results indicated that the R243Q mutation has a destabilizing effect on the N-terminal portion of the TRβ LBD, arising partially from the loss of a salt bridge and resulting in possible destabilization of the entire mutant LBD structure. Although this mutation does not abolish ligand-binding ability, it has been shown to decrease ligand-binding ability and increase co-repressor affinity. Finally, the P453H mutation resides outside the binding pocket on helix 12, which acts as a gatekeeper to the binding pocket and thus plays a critical role in the conformational changes associated with ligand binding (Rosen & Privalsky 2009, Souza et al. 2011). Native helix 12 has only one negatively charged residue, while all the other residues are nonpolar. Thus, the P453H mutation would change the electronic microenvironment and may impair the interaction between helix 12 and helix 3 in the presence of T3. This change would be consistent with the ~50-fold decrease in the observed binding affinity of this mutant for T3. Importantly, the observed relative decreases in T3-binding ability in the mutants investigated in the present study are consistent with the previously reported results using conventional direct binding methods (Table 3). For example, the reported in vitro binding assays indicate that the T3-binding affinity is only 10% of that of the WT in the R338W mutant, which is consistent with our bacterial sensor (0.13), while the Δ430M mutation has been reported to completely abolish T3-binding ability (Collingwood et al. 1994, Yoh et al. 1997). Furthermore, the reported decrease in T3-binding affinity arising from the P453H mutation is much greater (only 4% of that of the WT) (Cheng et al. 1994), while our system indicates binding activity at 2% of that of the WT.

Triac has been shown to have a higher affinity for human TR receptors than T3, although structural studies suggest that Triac is actually a worse fit for the TRβ ligand-binding pocket than T3 (Martinez et al. 2009). It has been proposed that the increased affinity of Triac for the TRβ LBD is due to entropic considerations, which complicates the analysis of mutation effects based on purely structural information. For example, the L346 residue does not directly interact with Triac, as it does with T3, but its neighboring residue G344 interacts with both T3 and Triac. This might explain why the L346F mutant recovers a positive growth phenotype in the presence of Triac, although the binding affinity is ~70 times lower than that of the native receptor. The ability of Triac to affect most of the mutants tested, and more than any other ligand in our library, supports the assertion that it is a potentially useful therapeutic for a wide range of RTH patients.

Compared with TRx, TRβ exhibits a higher affinity for GC-1 (approximately fivefold) and KB-141 (approximately tenfold). This selectivity has been attributed to the N331 residue, although structural and dynamic studies indicate that the interactions are indirect and rely heavily on the configurations and dynamics of the nearby arginine residues as well (Wagner et al. 2001, Grover et al. 2005,
Table 3  Comparison of the previously reported data and our biosensor data for selected mutants. For each mutant, the relative binding affinity for T3 is provided (as a percentage of that of the WT), along with the assay method and reference for the previously reported work. Although the absolute Ka values are very different from the EC50 values reported herein, the relative binding affinities are much closer.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>T3Ka M⁻¹</th>
<th>Relative binding (%WT)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R243Q</td>
<td>NR</td>
<td>0.21</td>
<td>Helix assembly assay</td>
<td>Huber et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>1.19 × 10⁻¹⁰</td>
<td>0.84</td>
<td>In vitro binding assay</td>
<td>Yagi et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>1.35 × 10⁻¹⁰</td>
<td>0.74</td>
<td>In vitro binding assay</td>
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<td></td>
<td>5.26 × 10⁻¹⁰</td>
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<td>7.69 × 10⁻⁶b</td>
<td>0.15</td>
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<td>This study</td>
</tr>
<tr>
<td>R338W</td>
<td>1.92 × 10⁻¹⁰</td>
<td>0.34</td>
<td>In vitro binding assay</td>
<td>Cheng et al. (1994)</td>
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<tr>
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<td>4.76 × 10⁻¹⁰</td>
<td>0.09</td>
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<td>Yoh et al. (1997)</td>
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<td>8.76 × 10⁻⁶⁰</td>
<td>0.13</td>
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<td>∆430M</td>
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<td>ND</td>
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<td>Yoh et al. (1997)</td>
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<td></td>
<td>ND</td>
<td>ND</td>
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<td>Collingwood et al. (1994)</td>
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<tr>
<td>P453H</td>
<td>6.67 × 10⁻¹⁰</td>
<td>0.06</td>
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<td>Collingwood et al. (1994)</td>
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<td>1.75 × 10⁻⁹</td>
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<td>1.96 × 10⁻¹⁰</td>
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<td>1.13 × 10⁻⁸a</td>
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<td>This study</td>
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</table>

*Data collected from only the LBD.

bRelative binding affinity is calculated based on the measured EC50 values in M.

Schriks et al. 2007, Bleicher et al. 2008). Interestingly, the binding affinities for GC-1 and KB-141 are not strongly affected by the R243W and R338W (group 1) mutations, and these mutations actually appear to increase binding affinities in some cases. The only other mutant that reacts to either of these compounds is P453H, which responds to GC-1 and KB-141 are much lower than those for T3 and Triac, but the binding affinities for GC-1 and KB-141 are not strongly affected by the R243W and R338W (group 1) mutations, and because there is no solved structure for TRβ in complex with NH-3, the source of this difference in binding cannot be examined through structural analysis. The results reported herein are consistent with indirect measurements, however, where studies have been carried out using hydrogen/deuterium exchange in TRβ in the presence of specific ligands (Lim et al. 2002). For example, Polikarpov et al. showed that helix 2 of the LBD exhibits far less deuterium exchange in the presence of T3 than does in the presence of NH-3 (0 vs 33%), while the β-sheet 4/helix 7 region of the LBD becomes equally substituted with both ligands (33 vs 33%) (Figueira et al. 2011). These observations imply that NH-3 may be more sensitive to mutations in helix 2 than T3, while both NH-3 and T3 may be sensitive to mutations in the β-sheet 4/helix 7 region. Our observations indicate that the R243W mutation, which is located on helix 2, leads to an observed threefold increase in NH-3-binding ability compared with the WT LBD. The R338W mutation, located adjacent to helix 7, has a much smaller impact relative to the WT sensor. These observations also indicate that although the NH-3 antagonist is a competitive binder to T3 in the LDB binding pocket, NH-3 is likely to interact with some residues that are different from those that interact with T3. The specific details of the competition mechanism will require more study.
Although our bacterial system is simpler and less expensive than most conventional assays, for many ligands it exhibits a significantly lower sensitivity. This is reflected in higher EC₅₀ values when compared with values obtained in conventional transcriptional assays and in vitro binding assays. There are several possible reasons for these disparities, including differences in ligand transport into the cells and the non-transcriptional nature of the assay (Skretas et al. 2007). For example, the bacterial assay relies on free diffusion or nonspecific transport of the ligands though the cell membrane, which will be differentially affected by the physical properties of each ligand. This may partially explain the large differences in EC₅₀ values between T₃ and Triac in the bacterial assay, where many conventional assays indicate a similar binding ability (Messier et al. 2001). In addition, the non-transcriptional nature of the bacterial system relies on stoichiometric binding of ligands to the expressed reporter proteins to produce the growth signal, while transcriptional assays produce multiple reporter gene transcripts through single binding events. This difference in mechanism may account for much of the decreased sensitivity of the bacterial system relative to transcriptional assays, but has proven advantageous for detecting low-affinity compounds (Skretas & Wood 2005a). These types of differences are inherent when comparing conventional assays that rely on completely different sensing and reporting mechanisms and typically lead to orders of magnitude differences in quantitative binding measurements from one assay to another. These differences can be greatly decreased by normalizing binding affinities within a single assay to calculate relative binding affinities, but even these strategies typically yield significant differences between assay types. This approach was applied to the binding of T₃ to the mutant receptors in the bacterial system and yielded values similar to those reported in several direct binding assays (Table 3). Thus, the bacterial system can provide consistent relative binding information for ligands and mutations, at least within the inherent variations observed in many conventional assays. Furthermore, the simplicity and low cost of the bacterial assay can provide significant advantages over many conventional transcriptional and direct binding methods.

In conclusion, we applied our bacterial hormone sensor system to study the effects of six mutations in the TRβ LDB observed in clinical RTH patients. The results indicate that our bacterial sensor system can provide some quantitative information on the relative effects of each mutation and our results are qualitatively consistent with those reported by previous investigators for these mutations. Furthermore, our sensor system can correctly identify NH-3 as a clear TRβ antagonist and indicate that it exhibits an increased ability to inhibit T₃ relative to the native receptor in at least one of the mutants studied. Our results indicate that the overall binding affinities for the native T₃ ligand are as follows: WT > R338W > R243W > P453H > L346F > N331D = δ430M, and those for the antagonist are as follows: R243W > WT = R338W. These results were obtained using a very simple and inexpensive assay, suggesting that this system may be used as an effective first screen for a wide range of mutant and ligand combinations. Ideally, this system can allow rapid prototyping of new mutant assay systems, with the goal of accelerating the evaluation of relatively safe thyromimetics for possible supplementation or rescue-mode applications in the early diagnosis of RTH patients.

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References
Cheng SY 2005 Thyroid hormone receptor mutations and disease: beyond thyroid hormone resistance. Trends in Endocrinology and Metabolism 16 176–182. (doi:10.1016/j.tem.2005.03.008)


Collingwood TN, Adams M, Tone Y & Chatterjee VK 1994 Spectrum of transcriptional, dimerization, and dominant-negative properties of 20 different mutant-thyroid-hormone β-receptors in thyroid-hormone resistance syndrome. Molecular Endocrinology 8 1262–1277. (doi:10.1210/me.8.9.1262)


Ocasio CA & Scanlan TS 2006 Design and characterization of a thyroid hormone receptor α (TRα)-specific agonist. ACS Chemical Biology 1 585–593. (doi:10.1021/cb603111v)


Reif Hoff AM & Dittmuseum AM 2007 Syndromes of reduced sensitivity to thyroid hormone: genetic defects in hormone receptors, cell transporters and deiodination. Best Practice & Research. Clinical Endocrinology & Metabolism 21 277–305. (doi:10.1016/j.beem.2007.03.003)


Rosen MD & Privalsky ML 2009 Thyroid hormone receptor mutations found in renal clear cell carcinomas alter corepressor release and reveal helix 12 as key determinant of corepressor specificity. Molecular Endocrinology 23 1183–1192. (doi:10.1210/mend.2009-0126)


Shuto Y, Wakabayashi I, Amuro N, Minami S & Okazaki T 1992 A point mutation in the 3,3’,5’-triiodothyronine-binding domain of thyroid-hormone receptor-β associated with a family with generalized...
resistance to thyroid-hormone. *Journal of Clinical Endocrinology and Metabolism* **75** 213–217. ([doi:10.1210/jc.75.1.213](http://dx.doi.org/10.1210/jc.75.1.213))


Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD & Fletterick Rj 1995 A structural role for hormone in the thyroid hormone receptor. *Nature* **378** 690–697. ([doi:10.1038/378690a0](http://dx.doi.org/10.1038/378690a0))

Wagner RL, Huber BR, Shiu A, Kelly A, Cunha,Alma ST, Scanlan TS, Apriletti JW, Baxter JD, West BL & Fletterick Rj 2001 Hormone selectivity in thyroid hormone receptors. *Molecular Endocrinology* **15** 398–410. ([doi:10.1210/me.15.3.398](http://dx.doi.org/10.1210/me.15.3.398))

Weiss RE, Weinberg M & Refetoff S 1993 Identical mutations in unrelated families with generalized resistance to thyroid-hormone occur in cytosine guanine-rich areas of the thyroid-hormone receptor-beta gene – analysis of 15 families. *Journal of Clinical Investigation* **91** 2408–2415. ([doi:10.1172/JCI116474](http://dx.doi.org/10.1172/JCI116474))

Yoh SM, Chatterjee VK & Privalsky ML 1997 Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T-3 receptors and transcriptional corepressors. *Molecular Endocrinology* **11** 470–480. ([doi:10.1210/me.11.4.470](http://dx.doi.org/10.1210/me.11.4.470))

Yagi H, Pohlhenz J, Hayashi Y, Sakurai A & Refetoff S 1997 Resistance to thyroid hormone caused by two mutant thyroid hormone receptor beta, R243Q and R243W, with marked impairment of function that cannot be explained by altered in vitro 3,5,3'-triiodothyronine binding affinity. *Journal of Clinical Endocrinology and Metabolism* **82** 1608–1614.


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