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Design and synthesis of complementing ligands for mutant thyroid hormone receptor TRβ(R320H): a tailor-made approach toward the treatment of resistance to thyroid hormone

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Abstract—The thyroid hormone receptors (TR) are ligand-dependant transcription factors that regulate key genes involved in metabolic regulation, thermogenesis and development. Resistance to thyroid hormone (RTH) is a genetic disease associated with mutations to TRβ that lack or show reduced responsiveness to thyroid hormone (triiodothyronine). Previously we reported that the neutral alcohol-based thyromimetic HY-1 can selectively restore activity to a functionally impaired form of TR associated with RTH without over-stimulating TRα, which has been associated with undesirable side effects. Two new series of tetrazole and thiazolidinedione based ligands were evaluated for their ability to recover potency and efficacy to three of the most common RTH-associated mutants, TRβ(R320C), TRβ(R320H), and TRβ(R316H), in cell based assays. A new thiazolidinedione based ligand **AH-9** was identified, which has near wild-type potency (EC_{50} = 0.54 nM) to TRβ(R320C) and TRβ(R320H). Significantly, **AH-9** is equipotent toward TRα(wt), TRβ(wt), TRβ(R320C), and TRβ(R320H), suggesting that **AH-9** may have the potential to restore the normal homeostatic balance of thyroid hormone actions in patients or models harboring these mutations.

1. Introduction

Resistance to thyroid hormone (RTH) is a genetic disease primarily caused by mutations to the ligand-binding domain of the thyroid hormone receptor, TRβ.[1,2] TRβ is a member of the nuclear hormone receptor superfamily of ligand-dependent transcription factors. RTH-associated TRβ mutants show diminished responsiveness to thyroid hormone (TH) at target tissues, leading to clinical presentations such as goiter, mental retardation, impaired bone maturation, and improper metabolism.

There exist two major forms of RTH: generalized resistance to thyroid hormone (GRTH) and pituitary resistance to thyroid hormone (PRTH).[2] Most GRTH patients have normal serum thyrotropin (TSH) levels and high levels of thyroid hormones (TH), T4 (tetraiodothyronine), and T3 (triiodothyronine). PRTH patients generally display both high TSH and high TH levels because the normal regulation of TH levels along the hypothalamus–pituitary axis is disrupted by an attenuated negative feedback response by TRβ (Fig. 1). In this case, exogenous TH may suppress the secretion of TSH and compensate the attenuated thyroid responsiveness

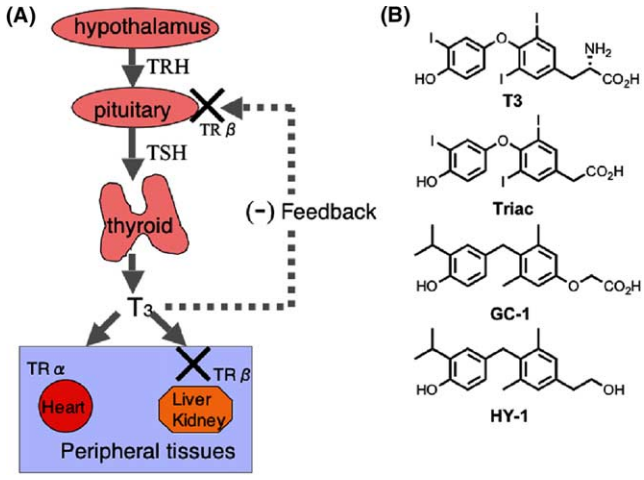

Figure 1. (A) TRβ is a key regulator of the hypothalamus–pituitaty axis. Mutant forms of TRβ associated with RTH disrupt the normal balance of TSH production and responsiveness of peripheral tissues. (B) Structures of T3, Triac, GC-1, and HY-1.

Keywords: Thyroid hormone receptor; Molecular complementation; Molecular rescue; RTH; Resistance to thyroid hormone.
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at peripheral tissues. However, high doses of T3 may lead to cardiotoxic side effects associated with over-stimulation of TRα, such as tachycardia and heart arrhythmia.[3] Ideally, hormone analogs that can specifically activate the mutant form of TRβ in both peripheral tissues and the hypothalamus may provide a novel therapeutic strategy for the treatment of RTH.[4–9]

The synthetic TR agonist, Triac (3,5,3′-triiodothyroacetic acid) has been proposed to have a unique potential for treating RTH symptoms such as elevated TSH and goiter because Triac binds wild-type TRβ (TRβ(wt)), more tightly than TRα(wt).[6,7] Triac also binds more tightly and is more active in cellular reporter gene assays than T3 toward some RTH associated mutants of TRβ. However, Triac is still more potent with wild-type TRs(TRα and TRβ) than mutant TRβ's suggesting that Triac may have only a limited benefit over T3 in restoring the normal homeostatic balance of TR function along the hypothalamic–pituitary–thyroid axis. For these reasons, the clinical use of Triac has been somewhat controversial.[10,11] Ideally, one would like to find T3 analogs that can activate the mutant and the wild-type TRβ without over-stimulating TRα(wt).

Our group has successfully developed hormone analogs that can rescue function to or 'complement' mutant nuclear hormone receptors associated with genetic disease.[8,9,12,13] Based on the co-crystal structures of the receptor ligand-binding domains with their natural hormones, we have designed small molecules that can selectively restore the activity to mutant forms of the thyroid hormone receptor, the estrogen receptor, and vitamin D receptor. Here we show that this approach can be applied to one of the most common TRβ mutants found among RTH patients, TRβ(R320H).

2. Results

2.1. Design strategy

The synthetic thyromimetic GC-1 was adopted as a structural scaffold for our designed mutant-targeting analogs because this high affinity halogen-free scaffold should be resistant to modification by cellular deiodinases.[14] The co-crystal structure of GC-1 with TRβ was also available as a guide for our ligand design.[15] The structure of the mutant receptor TRβ(R320H) has not been solved, but can be modeled. Schematic diagrams of the interactions of GC-1 with the wild-type receptor and the modeled RTH associated mutant TRβ(R320H) are depicted (Fig. 2A and B). In the complex with wild-type TRβ, GC-1 forms key polar interactions with His435 and a cluster of arginines (Arg282, Arg316, and Arg320) that electrostatically pair with the ligand's carboxylate. Compared to the wild-type receptor, the ligand binding pocket of the mutant receptor is enlarged by the Arg→His mutation. The substitution also causes a reduction in the receptor's electrostatic potential; the mutant receptor should show a diminished positive electrostatic character because histidine's pK_a (~6.5) is less than that of arginine (pK_a ~ 12).

Taking into account the differences in size and electrostatics of the TRβ(R320H) binding pocket, we used a bioisosteric replacement strategy to substitute the carboxylic acid of GC-1 with larger and more neutral functional groups. Although hundreds of thyroid hormone analogs and mimics have been reported, few thyromimetics have been reported that use a bioisosteric replacement of the carboxylate of T3.[12,13] The ligand-binding domain of TRβ(wt) uses a cluster of three arginines to bind carboxylic acids (pK_a ~ 3.1) with high affinity. The thyromimetic GC-1 is significantly less potent toward TRβ(R320H) (EC_{50} = 19.0 ± 4.0 nM) that has only two arginines than TRβ(wt) (EC_{50} = 3.6 ± 1.0 nM). We have shown that the GC-1 analog, HY-1, in which the carboxylic acid is replaced with a neutral alcohol, is more potent toward the RTH-associated mutant TRβ(R320C) than TRβ(wt).[9] Although HY-1 is less potent than T3 is with TRβ(wt), the mutant selectivity of HY-1 suggests that it may be uniquely suited to relieve the dominant negative actions of RTH-mutants while avoiding cardiotoxic side effects. We reasoned that analogs with functional groups of intermediate acidity (3.1 < pK_a's < 14) might be able to bind with high affinity to the mutant receptors that contain only two

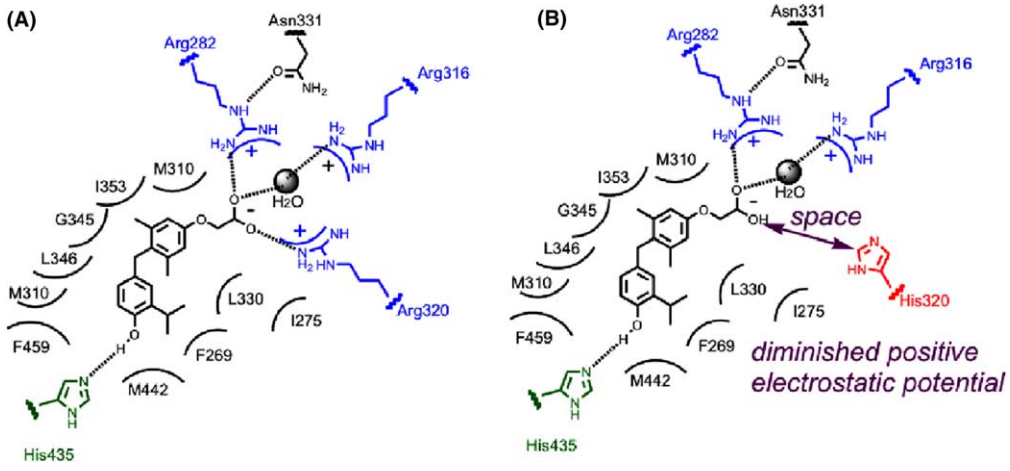
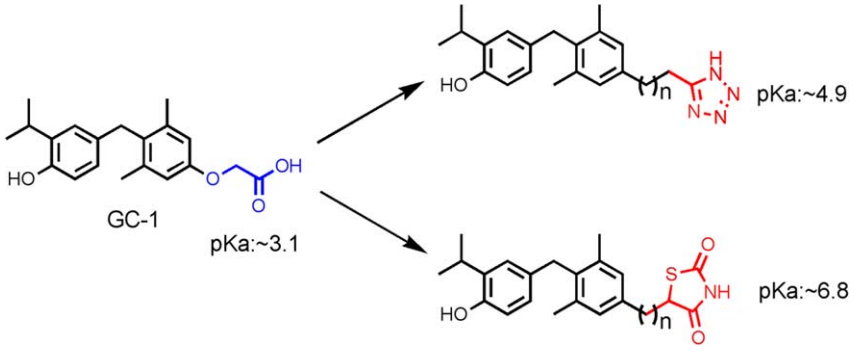


Figure 2. (A) Interactions of GC-1 with TRβ(wt). (B) Interaction of GC-1 of TRβ(R320H).



Scheme 1. Designed compounds based on bioisosteric replacement.

arginines in their binding sites. Among the common carboxylic acid bioisosters, tetrazoles ($pK_a = 4.9$) and thiazolidinediones ($pK_a = 6.8$) are less acidic than carboxyl groups ($pK_a \sim 3.1$) but more acidic than an alcohol. Additionally, these heterocycles are larger than the carboxylic group and may better complement the larger binding pocket of the Arg→His mutant. A series of tetrazole and thiazolidinedione based compounds containing different linker lengths were selected as target ligands (Scheme 1).

2.2. Synthesis

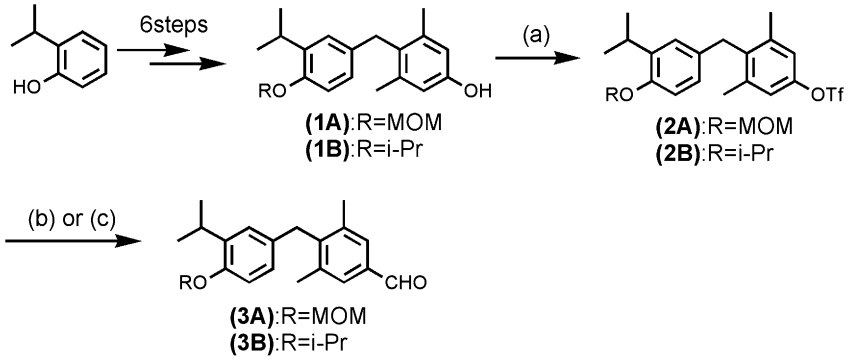
The intermediates (1A and 1B) were synthesized following reported methods and converted to the triflates (2A and 2B) (Scheme 2). The triflates were converted to the aldehydes by direct carbopalladation or a two step procedure, involving first introducing the vinyl group by the Stille coupling followed by ozonolysis to the aldehydes (3A and 3B).

The tetrazole analog **YS-1** was synthesized from intermediate (2B). The triflate (2B) was converted to nitrile by palladium cross-coupling reaction with zinc cyanide, followed by deprotection of isopropyl group and tetrazole cyclization reaction using sodium azide, giving **YS-1**. The other tetrazole analogs **YS-2** and **YS-3** were synthesized in the same procedure, from the corresponding nitrile, then deprotecting the isopropyl group followed by tetrazole (Scheme 3).

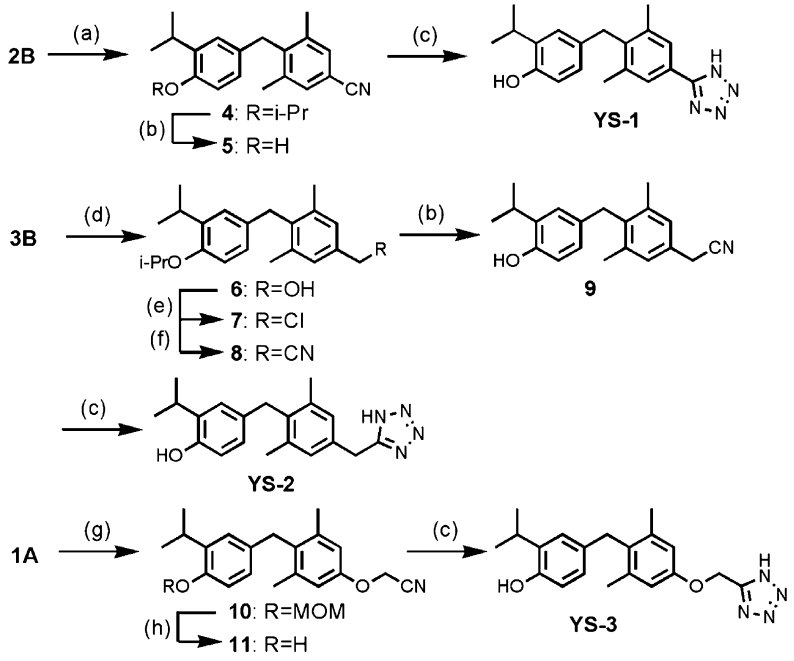
The analog **AH-21** was synthesized from intermediate **12** formed from the trimethylsilyl protected cyanohydrin, derived from the treatment of 3A with trimethylsilyl cyanide following alcoholysis under anhydrous acid. The α-hydroxy ester **12** was converted to the chloride by treatment with thionyl chloride, then cyclized with thiourea and hydrolyzed under acidic conditions to obtain **AH-21**.[16,17] The thiazolidinedione derivatives, **AH-7**, **AH-9**, and **AH-22** were also synthesized from **3A** by Knoevenagal condensation to form intermediate **13**. Intermediate **13** could be directly deprotected to give **AH-7**. Hydrogenation of **13** with palladium carbon to reduce the double bond was extremely sluggish presumably because the sulfur atom poisoned the catalyst. After screening several 1,4-reduction procedures, **13** was exclusively reduced to **14** in excellent yield using a catalytic amount of cobalt dichloride–dimethylglyoxime complex with excess sodium borohydride.[18] Deprotection of **14** afforded **AH-9** and methylation of **14** followed by deprotection of **15** afforded **AH-22** (Scheme 4).

2.3. Cellular activity

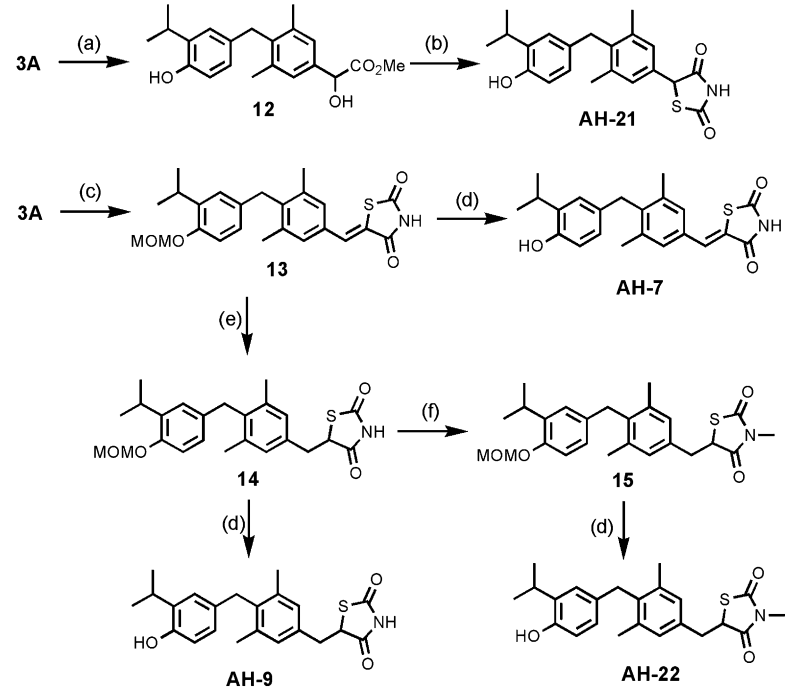
Each of the analogs were evaluated in cell-based reporter gene assays using a dual luciferase reporter gene assay (Promega). HEK293 cells were transiently co-transfected with pSG5hTRβ, DR4-tk-Luc+, and pRL-CMV by the $CaPO_4$ coprecipitate method. Full dose–response curves were established from three independent experiments each performed in triplicate to obtain the



Scheme 2. Synthesis of key intermediate. Reagents and conditions: (a) 4-nitrophenyl triflate, K_2CO_3, DMF, (b) trioctylsilane or triethylsilane, $Pd(OAc)_2$, TEA, dppp, CO(gas), DMF, (c) (1) vinyltributyltin, LiCl, $(Ph_3P)_2PdCl_2$, DMF, 60 °C; (2) O_3, CH_2Cl_2, −78 °C.



Scheme 3. Synthesis of tetrazole derivatives. Reagents and conditions: (a) $Zn(CN)_2$, CuI, $Pd(PPh_3)_4$, DMF, 90 °C, (b) $AlCl_3$, CH_2Cl_2, rt, (c) NaN_3, TEA–HCl, toluene, reflux, (d) $NaBH_4$, EtOH, rt, (e) $SOCl_2$, benzene, reflux, (f) KCN, KI, DMF, reflux, (g) bromoacetonitrile, Cs_2CO_3, DMF, rt, (h) 6-N HCl, MeOH, rt.

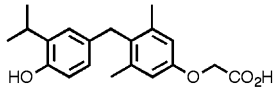
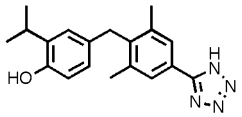
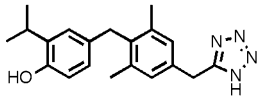
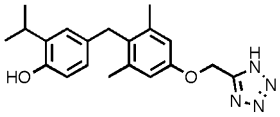
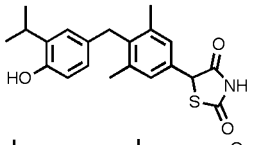
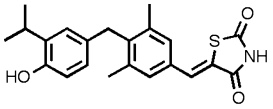
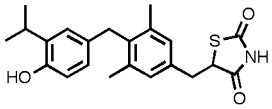
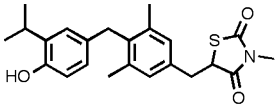


Scheme 4. Synthesis of thiazolidinedione analogs. Reagents and conditions: (a) (1) TMSCN, TEA, CH_2Cl_2, rt; (2) satd HCl–MeOH, Et_2O, rt, (b) (1) $SOCl_2$, pyridine, CH_2Cl_2, −78 °C to rt; (2) thiourea, AcONa, EtOH, reflux; (3) 2-N HCl, EtOH, (c) thiazolidinedione, piperidine, EtOH, reflux, (d) 5-N HCl, MeOH, rt, (e) $NaBH_4$, $CoCl_2$, DMG, 1-N NaOH, THF, 35 °C, (f) methyl iodide, K_2CO_3, acetone, reflux.

potency (EC_{50}) and efficacy (percent wild-type activity at saturation) (Table 1).

The cellular activities of the tetrazole derivatives **YS-1**, **YS-2**, and **YS-3**, suggest that the one atom linker of **YS-1** is inadequate for potent activation of the mutant or wild-type receptors. The best tetrazole analog **YS-3**, the direct tetrazole replacement of the carboxylate of GC-1, has good potency toward the mutant receptor TRβ(R320H) ($EC_{50} = 3.2 \pm 0.3$ nM), a significant improvement in potency over GC-1 ($EC_{50} = 19.0 \pm 4.0$ nM) for this mutant. However, **YS-3** is more potent

Table 1. EC_{50}s of tetrazole and thiazolidinedione analogs

		EC50(nM) /(efficacy, % max)		
		TRα	TRβ	R320H
			-5 fold	
GC-1		6.6±1.1 (110)	3.6±1.0 (100)	19.0±4.0 (82)
YS-1		-	180 ±10 (100)	280±60 (75)
YS-2		25.3±3.0 (100)	35.0±8.0 (100)	24.0 ±2.0 (75)
			-2 fold	
YS-3		1.4±0.2 (100)	1.6±0.2 (100)	3.2±0.3 (120)
			-4 fold	
AH-21		4.1±1.0 (90)	4.1±0.6 (100)	15.7±3.1 (100)
AH-7		65.1±13.9 (100)	52.0±7.7 (90)	80.1±3.5 (100)
			equal	
AH-9		0.47±0.13 (100)	0.54±0.07 (100)	0.46±0.05 (120)
AH-22		54.1±12.7 (85)	114.6±10.1 (100)	186.6±31.2 (80)

EC_{50} reported as the average of three independent experiments run in triplicate (±SEM). Efficacies, reported as percent of wild-type TR plus T3 activity at saturation indicated in parenthesis.

with TRβ(wt) (EC_{50} = 1.6 ± 0.2 nM) and is 2 times more potent than GC-1 with wild type. Therefore **YS-3** has the opposite mutant to wild-type selectivity (−2-fold) intended for potential RTH therapy, but represents a significant improvement in selectivity compared to GC-1 (−5-fold). Interestingly, although the oxygen of the oxoacetic acid side chain of GC-1 is believed to significantly contribute to GC-1's preference for TRβ over TRα, **YS-3** shows no beta-subtype selectivity.[19]

For the less acidic thiazolidinedione based analogs, **AH-21** (for which the thiazolidinedione is directly attached to the benzene ring) had relatively high potency with TRα(wt) and TRβ(wt). However, its mutant to wild-type selectivity was similar to that of GC-1. On the other hand, **AH-9** (which contains a single methylene linker to the thiazolidinedione) was highly potent (EC_{50} = 0.46 ± 0.05 nM) toward both the mutant TRβ(R320H) and TRβ(wt) (EC_{50} = 0.54 ± 0.07 nM). **AH-9** was 40 times more potent than GC-1 (EC_{50} = 19.0 ± 4.0 nM) toward TRβ(R320H) and had greater efficacy (120%) than GC-1 (82%).

The other thiazolidinedione analogs, **AH-7** (which contains a double bond linker) and **AH-22** (in which the nitrogen atom is masked with a methyl group) were much less potent. These results suggest that the thiazolidinedione ring needs to be attached to the benzene ring with some flexibility and requires an acidic proton to achieve high potency.

Encouraged by **AH-9**'s high potency toward both the mutant and the wild-type receptor, we also evaluated it in related receptors with RTH-associated mutations to the carboxylate-binding arginine cluster, TRβ(R320C) and TRβ(R316H) (Table 2). **AH-9** showed low potency toward TRβ(R316H) (EC_{50} = 105 ± 16.5 nM), but was

Table 2. EC_{50}s of T3, GC-1, and **AH-7**

	EC50(nM)/ (efficacy, %max)				
	TRα	TRβ	R320H	R320C	R316H
		-5 fold	-10 fold		-50 fold
T3	0.15±0.02 (110)	0.45±0.1 (100)	2.5±0.3 (70)	4.3±0.5 (72)	22.8±0.3 (50)
		-5 fold	-10 fold		-39 fold
GC-1	6.6±1.1 (100)	3.6±1.0 (100)	19.0±4.0 (82)	37.7±10.8 (72)	96.7±19 (50)
		equal	equal		-196 fold
AH-9	0.47±0.13 (100)	0.54±0.07 (100)	0.46±0.05 (120)	0.67±0.15 (120)	105.8±16.5 (110)

EC_{50} reported as the average of three independent experiments run in triplicate (± SEM). Efficacies, reported as percent of wild-type TR plus T3 activity at saturation, indicated in parenthesis.

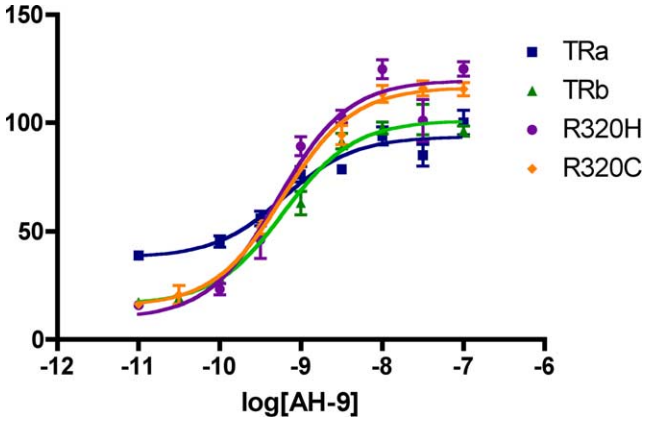


Figure 3. Dose–response curve of **AH-9** with wild-type and mutant TRs. Activity reported as relative light units from luciferase reporter gene assay.

highly effective in rescuing the potency and activity of TRβ(R320C) (max activity = 120%, EC_{50} = 0.67 ± 0.15 nM), with similar potency in TRα and TRβ(wt) (Fig. 3). The recently reported co-crystal structure of Triac with the mutant TRβ(316H) shows considerable structural disorder near helix-1 and a distorted binding pocket, which may account for **AH-9**'s reduced activity with this receptor.[20]

3. Discussion

The imbalanced response between TRα and TRβ caused by mutant TRβ's presents a major challenge in treating patients with RTH. Based on our understanding of the molecular basis of thyroid hormone action, hormone analogs, which complement mutant forms of TRβ associated with RTH should activate mutant TRβ with high potency and efficacy at concentrations that do not overstimulate TRβ(wt) or TRα(wt). Mutant-complementing analogs need not be as potent as T3 is with wild-type TRs so long as they have appropriate mutant versus wild-type selectivity. Since such mutant-selective complementing analogs have not previously been available and therefore have not been tested in vivo, it is unclear if the ideal complement needs to be selective for the mutant, or have equal potency in both mutant and wild type. We ultimately seek to develop compounds of both classes in order to test their efficacy in vivo models. In either case, it is desirable to have more potent and more specific analogs that may reduce the risk of potential side effects.

More than 100 different mutations have been identified in RTH patients. A priori one might assume that each mutant may require a unique complementing ligand. Furthermore, it is likely that for many mutant forms of TRβ, no ligand may be able to rescue receptor function. In this study we have focused on developing molecular complements for the Arg320→His mutation of TRβ because it is one of the most common mutations that have been identified in RTH patients. We have successfully identified compounds with promising activities for both TRβ(R320H) and the closely related mutant TRβ(R320C).

These mutations, which involve charged residues being converted to a neutral but polar residues, proved more challenging than previously studied mutations of VDR that involved charged to hydrophobic substitutions.[12,13] In prior studies we have found that neutral alcohols can selectively complement TRβ mutations that affect the carboxylate-binding arginine cluster by taking advantage of the unique differences in the receptor binding pocket's electrostatic environment compared to that of

wild type. The neutral alcohol HY-1 selectively activated mutant TRβ(R320C) over TRβ(wt). However, its potency toward the mutant TRβ(R320C) (EC_{50} = 7.0 ± 2.0 nM) was approximately 16 times less than T3's toward the wild-type TRβ (EC_{50} = 0.45 ± 0.1 nM).

In this study, we explore if the acidity of the side chain can be tuned such as to provide favorable selectivity while maintaining high potency using bioisosteric replacement of the carboxylate side chain of GC-1 for tetrazoles or thiazolidinediones. Both series of analogs improved selectivity between TRβ(wt) and TRβ(R320H) compared to the parent carboxyl-based analog GC-1, suggesting that, though also acidic, the less acidic tetrazoles and thiazolidinediones can provide a significant improvement over the parent carboxylic acid. The optimal ligands in both series significantly improved the potency compared to the more selective alcohol derivative HY-1, suggesting that for these mutations that affect the carboxylate-binding cluster, there is a balance between potency and selectivity that can be tuned by adjusting the pK_a of the ligand.

Molecular modeling suggests that the binding site of the TRβ(wt) is too small to accommodate the thiazolidinedione analog, **AH-9**. However, **AH-9** is still quite potent toward TRβ(wt), suggesting that the receptor has sufficient flexibility to accommodate this notably larger ligand without losing its ability to adopt a transcriptionally active conformation. The considerable plasticity of the nuclear receptor structure has been noted in other ligand–receptor engineering strategies and limits the application of steric-based or 'bump-and-hole' approaches to ligand–receptor engineering.[21] The development of strategies to selectively complement modified proteins using methods that are not solely steric-based is particularly important in NHRs and other highly flexible proteins. In this case **AH-9** is not only able to complement TRβ(R320H) but is also able to rescue the electrostatically similar TRβ(R320C) mutant with almost equal potency. **AH-9** was unable to rescue function to the related mutant TRβ(R316H), presumably because this mutation has been found to cause significant perturbations to the receptor structure. These findings illustrate that different but related mutations may be complemented by the same or similar ligands, suggesting that the chemical rescue of disease-associated mutations need not require a unique ligand for every mutation.

The thiazolidinedione analog, **AH-9**, has the optimal balance between potency and selectivity and can restore near wild-type potency (EC_{50} = 0.46 ± 0.05 nM) to TRβ(R320H) and more importantly normalizes the mutant and wild-type potency/activity (EC_{50}s: TRα, 0.47 ± 0.13 nM; TRβ, 0.54 ± 0.07 nM). In addition to being the most potent mutant-targeting thyromimetic yet reported, **AH-9** has the unique property of acting equally toward both the wild-type and mutant alleles in a nearly identical manner to that of T3 with wild-type TRα and TRβ. Therefore, in these initial in vitro findings suggest that **AH-9** can fully rescue TR/T3 function by restoring normal potency, activity, and selectivity.

4. Conclusion

The molecular complementation of RTH caused by dominant negative mutants of TRβ presents a unique challenge in molecular design because of the presence of two wild-type TR subtypes. Analogs that complement mutant forms of TRβ should not cause over-stimulation of TRα or TRβ wild type. Whether complementing analogs need to be selective or only equipotent in mutant and wild type has not yet been tested in vivo.

Mutations that affect the carboxylate-binding arginine cluster of TRβ are some of the most common mutants associated with RTH. Using a bioisosteric replacement strategy, one can adjust the acidity of the carboxylate side chain of the thyromimetic GC-1 to modulate the potency and selectivity of complementing analogs for TRβ(R320H); following a predictable trend, more acidic ligands favor binding the wild-type receptors, whereas less acidic ligands preferentially activate Arg320 mutants. The new mutant complementing analog **AH-9** can normalize both activity and potency of the wild-type receptors (TRα(wt) and TRβ(wt)) and mutant thyroid receptors (TRβ(R320H) and TRβ(R320C)) to the same level as T3 with wild-type TRs. The unique properties of **AH9** suggest that it may serve as a model for the chemical-rescue RTH caused by dominant negative mutants of TRβ.

5. Experimental

5.1. General

All compounds were purchased from Acros, Fisher Scientific International Inc. unless otherwise noted. NMR spectra were obtained using Bruker AMX 360 or DRX 400 MHz spectrophotometers at the University of Delaware NMR facility. Mass spectrometry was performed at the University of Delaware mass spectrometry laboratory. HEK 293 cells were obtained from ATTC (American Type Tissue Collection).

5.2. Synthesis

5.2.1. Trifluoro-methanesulfonic acid 4-(3-isopropyl-4-methoxymethoxy-benzyl)-3,5-dimethyl-phenyl ester (2A). Compound **1A** was synthesized by following the procedure described by Grazia et al.[22] To a solution of **1A** (5.0 g, 15 mmol) in 50 ml of DMF was added K_2CO_3 (4.2 g, 30 mmol). The mixture was stirred 10 min at room temperature before *p*-nitrophenyl triflate (4.50 g, 16.5 mmol) was added. After 4 h at room temperature, water (200 ml) and ether (75 ml) were added, and the mixture partitioned. The organic layer was washed with 1 N HCl (50 ml), 1 N NaOH (3 × 50 ml), water (50 ml), brine, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to afford 6.5 g (97%) of **2A**. ^{1}H NMR (400 MHz, $CDCl_3$) 6.95 (s, 2H), 6.88–6.92 (m, 2H), 6.58 (d, J = 8.4 Hz, 1H), 5.13 (s, 2H), 3.95 (s, 2H), 3.45 (s, 3H), 3.27 (m, 1H), 2.26 (s, 6H), 1.16 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 152.94, 147.66, 139.93, 138.16, 137.85, 131.65, 126.18,

125.52, 120.54, 114.18, 94.74, 63.35, 56.23, 34.24, 27.14, 23.01, 22.95, 20.64; HRMS calcd for $C_{21}H_{25}F_3O_5S$: 446.1375, found: 446.1370.

5.2.2. 4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-phenol (1B). Compound **1B** was synthesized by following the same procedure as preparation of compound **1A**. 5.65 g (0.012 mol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) 6.95 (s, 2H), 6.88 (s, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.55 (d, J = 8.4 Hz, 1H), 4.92 (br s, 1H), 4.45 (m, 1H), 3.94 (s, 2H), 3.25 (m, 1H), 2.26 (s, 6H), 1.28 (d, J = 6.0 Hz, 6H), 1.13 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.57, 153.18, 138.85, 137.94, 131.90, 130.00, 126.30, 125.20, 114.91, 113.18, 70.19, 33.86, 27.09, 22.95, 22.46, 20.53; HRMS calcd for $C_{21}H_{28}O_2$: 312.2089, found: 312.2085.

5.2.3. Trifluoro-methanesulfonic acid 4-(4-isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-phenyl ester (2B). Compound **2B** was synthesized by following the same procedure as preparation of compound **2A**. To a solution of **1B** (5.0 g, 15 mmol) in 50 ml of DMF was added K_2CO_3 (4.2 g, 30 mmol). The reaction mixture was stirred 10 min at room temperature, before *p*-nitrophenyl triflate (4.50 g, 16.5 mmol) was added. After 4 h at room temperature, water (200 ml) and ether (75 ml) were added, and the mixture was partitioned. The organic layer was washed with 1 N HCl (50 ml), 1 N NaOH (3 × 50 ml), water (50 ml), brine, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to give 6.5 g (98%). ^{1}H NMR (400 MHz, $CDCl_3$) 6.95 (s, 2H), 6.88 (s, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.55 (dd, J = 8.4, 2.3 Hz, 1H), 4.45 (m, 1H), 3.94 (s, 2H), 3.23 (m, 1H), 2.26 (s, 6H), 1.28 (d, J = 6.0 Hz, 6H), 1.13 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.55, 147.63, 139.95, 138.38, 138.20, 130.25, 126.28, 125.12, 120.43, 113.06, 70.07, 34.23, 27.11, 22.86, 22.43, 20.64; HRMS calcd for $C_{22}H_{27}F_3O_4S$: 444.1582, found: 444.1579.

5.2.4. 4-(3-Isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylbenzaldehyde (3A). Method (b): Palladium-catalyzed hydroformylation reaction. A mixture of compound **2** (676.2 mg, 1.5 mmol), $Pd(OAc)_2$ (10.0 mg, 0.045 mmol), 1,3-bis(diphenylphosphino)-propane (18.6 mg, 0.045 mmol), and triethylamine (374.4 mg, 3.7 mmol) in DMF (10 ml) was purged with carbon monoxide. Then, trioctylsilane (1.1 g, 3 mmol) was added in one portion and the reaction mixture was heated to 70 °C. After 3 h, the reaction mixture was diluted with water (20 ml) and EtOAc (60 ml). The organic layer was washed with brine (3 × 50 ml), satd $NaHCO_3$ (30 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography (hexane/EtOAc) to give 145.3 mg (30.0%) of **3A**.

5.2.4.1. Alternate method: Stille coupling followed by ozonolysis. To a stirred suspension of **2** (1.22 g, 2.74 mmol), lithium chloride (348.4 mg, 8.22 mmol), and dichlorobis(triphenylphosphine)palladium(96.2 mg, 0.14 mmol) in DMF (10 ml) was added vinyltributyltin (955.7 mg, 3 mmol) and the reaction mixture was stirred at 60 °C. After 1 h, the mixture was diluted with ether and hexane and aqueous saturated potassium fluoride was added and stirred for 1 h. Then, the mixture was filtered through a pad of Celite, which was rinsed with diethyl ether. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to a short silica gel column chromatography (hexane/EtOAc) to remove a polar component and after concentrated under reduced pressure, it was vacuumed to dry. The residue was then dissolved in methylene chloride (30 ml), cooled to −78 °C and bubbled with an oxygen/ozone mixture until the solution took on a light-violet color. The solution was sparged with nitrogen gas for 10 min and triphenylphosphine (2 g, 7.7 mmol) was added by one portion. The mixture was stirred for 30 min at room temperature and the solvent was removed under reduced pressure. The residue was purified by silica gel flash chromatography (hexane/EtOAc) to afford 479.0 mg (54%) of **3A**.

^{1}H NMR (400 MHz, $CDCl_3$) 9.95 (s, 1H), 6.89–6.93 (m, 2H), 7.57 (s, 2H), 6.61 (dd, J = 8.4, 2.2 Hz, 1H), 5.15 (s, 2H), 4.04 (s, 2H), 3.47 (s, 3H), 3.29 (t, J = 6.9 Hz, 1H), 2.23 (s, 6H), 1.18 (d, J = 6.9 Hz, 6H); ^{13}C NMR (90 MHz, $CDCl_3$) 192.54, 152.72, 144.92, 138.13, 137.63, 134.46, 131.26, 129.39, 125.96, 125.27, 113.98, 94.51, 55.96, 34.81, 26.93, 22.71, 20.19; HRMS calcd for $C_{21}H_{26}O_3$: 326.1882 (M+), found: 326.1885.

5.2.5. 4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-benzaldehyde (3B). A solution of triflate **5** (450 mg, 1.0 mmol) in 5 ml of DMF was sparged with nitrogen for 10 min before, palladium acetate (6.6 mg, 0.03 mmol) and bis(diphenylphosphino)-propane (dppp, 12 mg, 0.03 mmol) were added. The reaction mixture was heated to 70 °C. Carbon monoxide was rapidly bubbled through the yellow-brown solution until the color changed to black-brown. The carbon monoxide flow rate was slowed and the reaction mixture was heated for a five additional minutes. Triethylamine (0.56 ml, 4 mmol) was added dropwise via syringe followed by the slow addition of triethylsilane (0.32 ml, 2 mmol) via syringe over 20 min. The reaction was stirred at 70 °C overnight, then cooled to room temperature, diluted with ether (20 ml) and partitioned. The organic layer was washed with 1 N HCl (10 ml), water (2 × 20 ml), brine (20 ml), dried over magnesium sulfate, concentration under reduced pressure, and purified by silica gel column chromatography (hexane/EtOAc, 9:1) to afford 210 mg (0.65 mmol, 65%) of **3B**. ^{1}H NMR (400 MHz, $CDCl_3$) 9.93. (s, 1H), 7.55 (s, 2H), 6.89 (s, 1H), 6.66 (d, J = 8.4 Hz, 1H), 6.55 (dd, J = 8.4, 2.4 Hz, 1H), 4.44 (m, 1H), 4.01 (s, 2H), 3.24 (m, 1H), 2.31 (s, 6H), 1.28 (d, J = 6.0 Hz, 6H), 1.12 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 192.86, 145.45, 138.40, 138.21, 134.64, 129.64, 126.38, 125.19, 113.05, 70.05, 35.04, 27.11, 22.87, 22.42, 20.45; HRMS calcd for $C_{22}H_{28}O_2$: 324.2089, found: 324.2086.

5.2.6. 4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-benzonitrile (4). A solution of **2B** (445 mg, 1.0 mmol), copper(I) iodide (20 mg, 0.1 mmol), and zinc cyanide

(235 mg, 2 mmol) in 5 ml of DMF was sparged with nitrogen for 10 min before, 60 mg (0.05 mmol) of tetrakis(triphenylphosphine) palladium(0) was added. The reaction mixture was heated at 90 °C for 4 h, then cooled to room temperature, diluted with methylene chloride (20 ml), and washed with 1 N HCl (10 ml), water (2 × 20 ml), brine (20 ml), dried over magnesium sulfate, concentration under reduced pressure, and purified by silica gel column chromatography (hexane/EtOAc, 7:3) to give 275 mg (0.85 mmol, 85%) of **4**. ^{1}H NMR (400 MHz, $CDCl_3$) 7.34 (s, 2H), 6.90 (s, 1H), 6.68 (d, J = 8.4 Hz, 1H), 6.55 (dd, J = 8.4, 2.3 Hz, 1H), 4.47 (m, 1H), 4.00 (s, 2H), 3.27 (m, 1H), 2.28 (s, 6H), 1.31 (d, J = 6.0 Hz, 6H), 1.16 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.56, 143.66, 138.64, 138.21, 133.96, 131.53, 131.50, 129.40, 126.25, 125.08, 119.51, 112.99, 109.85, 69.96, 34.74, 27.08, 22.80, 22.34, 20.24; HRMS calcd for $C_{22}H_{27}NO$: 321.2093, found: 321.2092.

5.2.7. 4-(4-Hydroxy-3-isopropyl-benzyl)-3,5-dimethyl-benzonitrile (5). To a solution of **4** (250 mg, 0.8 mmol) in dichloromethane (3 ml) was added anhydrous $AlCl_3$ (140 mg, 1.0 mmol). The reaction mixture was stirred at room temperature overnight, diluted with water (10 ml), and extracted with EtOAc (2 × 10 ml). The combined organic extract was washed with brine (20 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to afford 150 mg (80%) of **5**. ^{1}H NMR (400 MHz, $CDCl_3$) 7.33 (s, 2H), 6.86 (s, 1H), 6.59 (dd, J = 8.4, 3.6 Hz, 1H), 6.49 (d, J = 8.4 Hz, 1H), 4.60 (br s, 1H), 3.97 (s, 2H), 3.13 (m, 1H), 2.25 (s, 6H), 1.18 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 151.35, 143.53, 138.69, 138.18, 131.65, 130.29, 126.35, 125.47, 115.56, 34.77, 27.31, 22.72, 20.30; HRMS calcd for $C_{19}H_{21}NO$: 279.1623, found: 279.1620.

5.2.8. 4-[2,6-Dimethyl-4-(1*H*-tetrazol-5-yl)-benzyl]-2-isopropyl-phenol (YS-1). A mixture of **5** (140 mg, 0.5 mmol), sodium azide (1.5 mmol), and triethylamine hydrochloride (1.5 mmol) in toluene (5 ml) was heated to reflux for 24 h. After cooling to room temperature, the product was extracted with water (5 ml). To the aqueous layer, concentrated HCl was added dropwise to salt out the product tetrazole **YS-1** (140 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.92 (s, 1H), 7.31 (s, 1H), 6.88 (s, 1H), 6.68 (dd, J = 8.0, 4.8 Hz, 1H), 6.46 (d, J = 8.4 Hz, 1H), 3.95 (s, 2H), 3.13 (m, 1H), 2.23 (s, 6H), 1.17 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 158.23, 151.64, 141.60, 138.68, 138.47, 131.59, 130.56, 127.29, 126.35, 126.18, 125.40, 115.65, 115.57, 34.63, 27.25, 22.75, 20.27; HRMS calcd for $C_{19}H_{22}N_4O$: 322.1794, found: 322.1789.

5.2.9. [4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-phenyl]-methanol (6). To a solution of **3B** (100 mg, 0.3 mmol) in ethanol (5 ml), was added three portions of $NaBH_4$ (50 mg, 1.3 mmol). The mixture was stirred at room temperature for 1 h before water (10 ml) was slowly added. The mixture was extracted with ethyl acetate (20 ml) and the organic layer was washed with 1 N HCl (10 ml), water (2 × 10 ml), brine (10 ml), dried over magnesium sulfate, and concentration under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc, 8:2) to afford **6** (100 mg, 0.3 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.06 (s, 2H), 6.98 (s, 1H), 6.68 (s, J = 8.4 Hz, 1H), 6.60 (dd, J = 8.4 Hz and 2.4 Hz, 1H), 4.62 (s, 2H), 4.45 (m, 1H), 3.98 (s, 2H), 3.28 (m, 1H), 2.27 (s, 6H), 1.30 (d, J = 6.0 Hz, 6H), 1.17 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.24, 138.61, 137.92, 137.61, 137.20, 131.20, 127.01, 126.42, 125.18, 113.04, 70.03, 65.45, 34.43, 27.11, 22.90, 22.41, 20.43; HRMS calcd for $C_{22}H_{30}O_2$: 326.2246, found: 326.2245.

5.2.10. 4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-benzyl chloride (7). To a solution of **6** (100 mg, 0.3 mmol) in benzene (10 ml) was added thionyl chloride (0.5 ml, 6.8 mmol). The reaction was heated to reflux for 2 h and the solvent and excess thionyl chloride were removed by evaporated under reduced pressure. The product **7** was used to the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) 7.09 (s, 2H), 6.96 (s, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.66 (dd, J = 8.4, 2.4 Hz, 1H), 4.55 (s, 2H), 4.46 (m, 1H), 3.97 (s, 2H), 3.28 (m, 1H), 2.26 (s, 6H), 1.30 (d, J = 6.0 Hz, 6H), 1.17 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.32, 138.21, 137.96, 137.89, 137.83, 135.17, 130.90, 128.47, 126.45, 125.18, 113.01, 70.01, 46.67, 34.47, 27.13, 22.90, 22.43, 20.42; HRMS calcd for $C_{22}H_{29}ClO$: 344.1907, found: 344.1903.

5.2.11. [4-(4-Isopropoxy-3-isopropyl-benzyl)-3,5-dimethyl-phenyl]-acetonitrile (8). A mixture of **7** (100 mg, 0.3 mmol), KCN (66 mg, 10 mmol), and KI (10 mg) in 5 ml of DMF was refluxed for 12 h. After cooling to room temperature, water (10 ml) was added and the mixture was extracted with ethyl acetate (20 ml). The organic layer was washed with water (2 × 10 ml), brine (10 ml), dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc, 9:1) to give 81 mg (0.24 mmol, 81%) of **8**. ^{1}H NMR (400 MHz, $CDCl_3$) 7.00 (s, 2H), 6.93 (s, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.57 (dd, J = 8.4, 2.4 Hz, 1H), 4.46 (m, 1H), 3.95 (s, 2H), 3.67 (s, 2H), 3.26 (m, 1H), 2.25 (s, 6H), 1.30 (d, J = 6.0 Hz, 6H), 1.16 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 153.35, 138.33, 138.00, 137.67, 130.75, 127.68, 127.44, 126.36, 125.13, 118.49, 113.02, 70.01, 34.32, 27.11, 23.33, 22.89, 22.41, 20.39; HRMS calcd for $C_{23}H_{29}NO$: 335.2249, found: 335.2248.

5.2.12. [4-(4-Hydroxy-3-isopropyl-benzyl)-3,5-dimethyl-phenyl]-acetonitrile (9). The same procedure was used for removal of isopropyl protecting group as preparation of compound **5**. ^{1}H NMR (400 MHz, $CDCl_3$) 6.99 (s, 2H), 6.91 (s, 1H), 6.59 (d, J = 8.4 Hz, 1H), 6.52 (d, J = 8.4 Hz, 1H), 4.81 (br s, 1H), 3.94 (s, 2H), 3.68 (s, 2H), 3.15 (m, 1H), 2.23 (s, 6H), 1.20 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 151.15, 138.33, 137.53, 134.55, 131.41, 127.71, 127.66, 127.46, 126.37, 125.46, 122.95, 115.40, 34.29, 27.27, 23.33, 22.74, 20.41; HRMS calcd for $C_{20}H_{23}NO$: 293.1780, found: 293.1776.

5.2.13. 4-[2,6-Dimethyl-4-(1*H*-tetrazol-5-ylmethyl)-benzyl]-2-isopropyl-phenol (YS-2). The same procedure was used as for preparation of compound **YS-1** (95%).

^{1}H NMR (400 MHz, $CDCl_3$) 7.01 (s, 2H), 6.93 (s, 1H), 6.60 (d, J = 8.4 Hz, 1H), 6.51 (d, J = 8.4 Hz, 1H), 4.91 (bs, 1H), 3.96 (s, 2H), 3.73 (s, 2H), 3.15 (m, 1H), 2.25 (s, 6H), 1.21 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.75, 143.94, 137.62, 138.13, 134.41, 131.37, 129.72, 127.53, 126.20, 125.40, 115.22, 56.97, 34.10, 27.11, 22.51, 20.16 HRMS calcd for $C_{20}H_{24}N_4O$: 336.1950, found: 336.1944.

5.2.14. [4-(3-Isopropyl-4-methoxymethoxy-benzyl)-3,5-dimethyl]-phenoxy]-acetonitrile (10). To a solution of **1A** (160 mg, 0.5 mmol) in 5 ml of DMF was added Cs_2CO_3 (370 mg, 1.1 mmol). The reaction mixture was stirred for 10 min at room temperature before bromoacetonitrile (0.1 ml, 1.4 mmol) was added. The reaction mixture was stirred at room temperature until the starting material disappeared and then water (20 ml) and ether (10 ml) were added and partitioned. The organic portion was washed with water (20 ml), brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica flash chromatography (hexane/ethyl acetate 90/10) to afford 105 mg (70%) of **10**.

^{1}H NMR (400 MHz, $CDCl_3$) 6.96 (s, 1H), 6.91 (d, J = 8.4 Hz, 1H), 6.70 (s, 2H), 6.65 (d, J = 8.4 Hz, 1H), 5.16 (s, 2H), 4.76 (s, 2H), 3.95 (s, 2H), 3.49 (s, 3H), 3.27 (m, 1H), 2.25 (s, 6H), 1.19 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 154.57, 152.54, 139.00, 138.58, 137.48, 132.70, 132.24, 129.39, 125.99, 115.54, 114.76, 113.95, 94.64, 56.01, 53.62, 33.82, 26.99, 22.83, 20.56; HRMS calcd for $C_{22}H_{27}NO_3$: 353.1991. found: 353.1985.

5.2.15. [4-(4-Hydroxy-3-isopropyl-benzyl)-3,5-dimethyl]-phenoxy]-acetonitrile (11). To a solution of **10** (50 mg, 0.15 mmol) in methanol (4 ml) was added 4 ml of 6 N HCl. The reaction mixture was stirred at room temperature overnight, diluted with water (10 ml), and extracted with ethyl acetate (2 × 20 ml). The combined organic extract was washed with brine (20 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give 32 mg (65%) of **11**. ^{1}H NMR (400 MHz, $CDCl_3$) 6.81 (s, 1H), 6.72 (s, 2H), 6.67 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 8.4 Hz, 1H), 4.73 (s, 2H), 3.94 (s, 2H), 3.26 (m, 1H), 2.25 (s, 6H), 1.19 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) 156.46, 152.51, 139.37, 138.85, 136.12, 133.36, 132.70, 132.24, 129.39, 125.99, 115.54, 114.13, 53.62, 33.82, 26.99, 22.83, 20.56; HRMS calcd for $C_{20}H_{23}NO_2$: 309.1729, found: 309.1722.

5.2.16. 4-[2,6-Dimethyl-4-(1*H*-tetrazol-5-ylmethoxy)-benzyl]-2-isopropyl-phenol (YS-3). The same procedure was used as for preparation of compound **YS-1**.

^{1}H NMR (400 MHz, CD_3OD) 6.77 (d, J = 2.0 Hz, 2H), 6.72 (s, 2H), 6.56 (d, J = 8.0 Hz, 1H), 6.49 (d, J = 8.0 Hz, 1H), 5.38 (s, 2H), 3.84 (s, 2H), 3.21 (m, 1H), 2.16 (s, 6H), 1.09 (d, J = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) 157.08, 153.54, 139.83, 135.92, 132.83, 131.86, 126.66, 126.42, 115.95, 115.31, 60.81, 34.59, 28.08, 23.18, 20.69; HRMS calcd for $C_{20}H_{24}N_4O_2$: 352.1899, found: 352.1897.

5.2.17. Methyl 2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dimethylphenyl)-2-hydroxyacetate (12). To a stirred solution of **3A** (715 mg, 2.19 mmol) and trimethylsilyl cyanide (340 mg, 3 mmol) in CH_2Cl_2 (20 ml) at 0 °C was added triethylamine (0.03 ml, 0.2 mmol). The reaction mixture was warmed up to room temperature and stirred for 12 h before being concentrated under reduced pressure. The residue was redissolved in ether (8 ml) and saturated methanolic HCl (30 ml) and the solution stirred at room temperature for 2 days. The mixture was concentrated under reduced pressure and the residue dissolved in CH_2Cl_2 (80 ml), washed with brine (30 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc) to afford **12** (519.9 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.25 (s, 2H), 6.93 (s, 1H), 6.51–6.57 (m, 2H), 5.11 (d, J = 5.7 Hz, 1H), 4.75 (br, 1H), 3.95 (s, 2H), 3.78 (s, 3H), 3.41 (d, J = 5.7 Hz, 1H), 3.15 (t, J = 6.9 Hz, 1H), 2.24 (s, 6H), 1.20 (d, J = 6.9 Hz); ^{13}C NMR (90 MHz, $CDCl_3$) 174.41, 150.85, 137.96, 135.71, 134.24, 131.38, 126.29, 125.37, 115.13, 72.87, 53.05, 34.25, 27.09, 22.53, 20.29; HRMS calcd for $C_{21}H_{26}O_4$: 342.1831 (M+), found: 342.1835.

5.2.18. 5-(4-(4-Hydroxy-3-isopropylbenzyl)-3,5-dimethylphenyl)thiazolidine-2,4-dione (AH-21). To a stirred solution of **12** (60 mg, 0.175 mmol) and pyridine (35 mg, 0.44 mmol) in CH_2Cl_2 (5 ml) at −78 °C was added dropwise thionyl chloride (44 mg, 0.37 mmol). The reaction mixture was warmed to room temperature and stirred for 12 h and concentrated under reduced pressure. The residue, thiourea (13.7 mg, 0.18 mmol), and sodium acetate (30 mg, 0.36 mmol) were dissolved in EtOH (4 ml) and the reaction mixture was refluxed for 4 h. After cooling to room temperature, the mixture was filtered through a pad of Celite, which was rinsed with ether. The combined filtrate was concentrated under reduced pressure. The residue was dissolved in EtOH (5 ml) and 2-N HCl (4 ml) and refluxed for 18 h. The solution was concentrated under reduced pressure. The residue was diluted with EtOAc (80 ml) and washed with brine (30 ml), dried over $MgSO_4$, concentrated under reduced pressure, and purified by silica gel column chromatography to give **AH-21** (24.4 mg, 38%).

^{1}H NMR (400 MHz, $CDCl_3$) 7.08 (s, 2H), 6.94 (s, 1H), 6.48–6.59 (m, 2H), 5.30 (s, 1H), 3.95 (s, 2H), 3.15 (t, J = 6.9 Hz, 1H), 2.25 (s, 6H), 1.22 (d, J = 6.9 Hz); ^{13}C NMR (90 MHz, $CDCl_3$) 173.38, 170.34, 150.91, 138.90, 138.42, 134.34, 131.06, 127.76, 126.32, 126.22, 125.31, 115.23, 54.94, 34.26, 27.16, 22.52, 20.27; HRMS calcd for $C_{21}H_{23}NO_3S$: 392.1296 (M+Na), found: 392.1284.

5.2.19. (*Z*)-5-(4-(3-Isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylbenzylidene)thiazolidine-2,4-dione (13). The reaction mixture of **3A** (112.6 mg, 0.35 mmol), 2,4-thiazolidinedione (43.6 mg), and piperidine (27 mg, 0.32 mmol) in EtOH (10 ml) was refluxed for 10 h. Then, the mixture was diluted with water (20 ml) and EtOAc (100 ml), washed with brine (50 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **13** (120.0 mg, 82%).

^{1}H NMR (400 MHz, $CDCl_3$) 7.82 (s, 1H), 7.20 (s, 2H), 6.89–6.95 (m, 2H), 6.61 (dd, J = 8.4, 2.2 Hz, 1H), 5.16 (s, 2H), 4.01 (s, 2H), 3.47 (s, 3H), 3.29 (t, J = 6.8 Hz, 1H), 2.30 (s, 6H), 1.18 (d, J = 6.8 Hz, 6H); ^{13}C NMR (90 MHz, $CDCl_3$) 167.56, 166.86, 152.72, 141.26, 138.32, 137.65, 134.81, 131.59, 130.75, 130.14, 126.06, 125.27, 120.98, 114.02, 94.54, 55.99, 34.63, 26.98, 22.76, 20.38; HRMS calcd for $C_{24}H_{27}NO_4S$: 448.1559 (M+Na), found: 448.1557.

5.2.20. (*Z*)-5-(4-(4-Hydroxy-3-isopropylbenzyl)-3,5-dimethylbenzylidene)thiazolidine-2,4-dione (AH-7). To a stirred solution of **13**(58.9 mg, 0.14 mmol) in EtOH (8 ml) and CH_2Cl_2 (2 ml) was added 5-N HCl (3 ml). The mixture was stirred at room temperature for 18 h and then concentrated under reduced pressure. The residue taken up in EtOAc (60 ml) and water (30 ml) and the layers partitioned. The organic layer was washed with brine (30 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **AH-7** (45.5 mg, 85%).

^{1}H NMR (400 MHz, $CDCl_3$) 7.79 (s, 1H), 7.20 (s, 2H), 6.92 (d, J = 1.9 Hz 1H), 6.59 (d, J = 8.2 Hz, 1H), 6.52 (dd, J = 8.2, 2.1 Hz, 1H), 3.99 (s, 2H), 3.18 (t, J = 6.9 Hz, 1H), 2.30 (s, 6H), 1.20 (d, J = 6.9 Hz, 6H); ^{13}C NMR (90 MHz, $CDCl_3$) 175.98, 167.83, 166.95, 141.29, 138.28, 134.46, 130.74, 130.29, 130.08, 126.19, 125.20, 121.16, 115.16, 115.04, 34.55, 27.01, 22.52, 20.35; HRMS calcd for $C_{22}H_{23}NO_3S$: 404.1296 (M+Na), found: 404.1286.

5.2.21. 5-(4-(3-Isopropyl-4-(methoxymethoxy)benzyl)-3,5-dimethylbenzyl)thiazolidine-2,4-dione (14). To a rapidly stirred mixture of **13** (663.2 mg, 1.56 mmol) in THF (6.8 ml) and 1-N NaOH at room temperature was added (1.7 ml), $CoCl_2$–DMG complex DMF solution (0.34 ml, made from 21 mg of cobalt chloride hexahydrate, 125 mg of dimethylglyoxime, and 2.5 ml of DMF). After 30 min, sodium borohydride (340 mg, 9.0 mmol) in water (7 ml) was added and the mixture was stirred at 35 °C for 3 h. Then, the reaction mixture was neutralized by 1-N NaOH, diluted with EtOAc (80 ml), and the organic layer was washed with brine (30 ml), and dried over $MgSO_4$. After concentrated under reduced pressure, the residue was purified by silica gel column chromatography to give **14** (613.3 mg, 92%).

^{1}H NMR (400 MHz, $CDCl_3$) 8.50 (br, 1H), 6.88–6.92 (m, 4H), 6.61 (d, J = 8.1 Hz, 1H), 5.15 (s, 2H), 4.54 (dd, J = 10.4, 3.7 Hz, 1H), 3.95 (s, 2H), 3.53 (dd, J = 14.0, 3.3 Hz, 1H), 3.47 (s, 3H), 3.28 (t, J = 6.8 Hz, 1H), 2.98–3.04 (m, 1H), 2.23 (s, 6H), 1.17 (d, J = 6.8 Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$) 174.16, 170.51, 152.53, 137.73, 137.44, 136.83, 133.61, 132.47, 128.63, 126.02, 125.30, 113.95, 94.59, 55.99, 53.74, 38.45, 34.18, 26.92, 22.79, 20.28; HRMS calcd for $C_{24}H_{29}NO_4S$: 427.1817 (M+), found: 427.1816.

5.2.22. 5-(4-(4-Hydroxy-3-isopropylbenzyl)-3,5-dimethylbenzyl)thiazolidine-2,4-dione (AH-9). To a stirred solution of **14** (42 mg, 0.1 mmol) in MeOH (10 ml) and CH_2Cl_2 (0.5 ml), 5-N HCl (2 ml) was added and stirred at room temperature for 18 h. Then, the reaction mixture was concentrated under reduced pressure, diluted with water (30 ml) and EtOAc (2 × 60 ml) and the organic layer was washed with brine (30 ml), and dried over $MgSO_4$. After concentrated under reduced pressure, the residue was purified by silica gel column chromatography to give **AH-9** (38 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) 8.32 (br, 1H), 6.91 (s, 3H), 6.52–6.60 (m, 2H), 4.54 (dd, J = 10.4, 3.8 Hz, 1H), 3.94 (s, 1H), 3.53 (dd, J = 14.0, 3.8 Hz, 1H), 3.15 (t, J = 6.9 Hz, 1H), 3.02 (dd, J = 14.0, 10.4 Hz, 1H), 2.22 (s, 6H), 1.20 (d, J = 6.9 Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$) 174.10, 170.47, 150.80, 137.71, 136.88, 134.24, 133.53, 131.53, 128.63, 126.18, 125.33, 115.17, 53.70, 38.40, 34.11, 27.05, 22.53, 20.24; HRMS calcd for $C_{22}H_{25}NO_3S$: 406.1453 (M+Na), found: 406.1433.

5.2.23. 5-(4-(4-Hydroxy-3-isopropylbenzyl)-3,5-dimethylbenzyl)-3-methylthiazolidine-2,4-dione (AH-22). To a stirred suspension of **14** (64 mg, 0.15 mmol) and potassium carbonate (41.5 mg, 0.3 mmol) in acetone (10 ml) was added methyl iodide (24.1 mg, 0.17 mmol). The reaction mixture was heated to reflux for 2 h, then cooled and filtered through a pad of Celite, which was rinsed with ether. The filtrate was washed with brine (40 ml), dried over $MgSO_4$, and concentrated under reduced pressure to afford **15**, which was used subsequently without further purification. To a solution of **15** was in MeOH (10 ml) and CH_2Cl_2 (0.5 ml) was added 5-N HCl (2 ml). The reaction mixture was stirred at room temperature for 18 h then concentrated under reduced pressure. The residue was taken up in EtOAc (60 ml) with water (30 ml) and partitioned. The organic layer was washed with brine (30 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give **AH-22** (41.8 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) 6.90 (s, 3H), 6.51–6.60 (m, 2H), 4.73 (br, 1H), 4.46 (dd, J = 10.4, 3.7 Hz, 1H), 3.93 (s, 2H), 3.55 (dd, J = 13.9, 5.0 Hz, 1H), 3.16 (t, J = 6.9 Hz, 1H), 2.95 (dd, J = 13.9, 10.4 Hz, 1H), 2.22 (s, 6H), 1.20 (d, J = 6.9 Hz, 6H); ^{13}C NMR (90 MHz, $CDCl_3$) 174.22, 171.43, 150.83, 137.65, 136.80, 134.26, 133.72, 131.57, 128.65, 126.19, 125.35, 115.19, 51.96, 38.64, 34.12, 27.82, 27.09, 22.55, 20.22; HRMS calcd for $C_{23}H_{27}NO_3S$: 420.1600 (M+Na), found: 420.1600.

5.3. Plasmid constructs

Plasmid pSG5-TRβ mutants R320C, R320H, and R316H were constructed from the parent plasmid pSG5-TRβ by oligonucleotide-directed mutagenesis

using Quickchange (Stratagene) using the following oligonucleotides:

R320Cf: CGCGCTGCTGTGTGCTATGACCCGGAAAG
R320Cr: CTTTCCGGGTCATAGCACACAGCAGCGCG
R320Hf: CGCGCTGCTGTGCACTATGACCCGGAAAGTG
R320Hr: CACTTTCCGGGTCATAGTGCACAGCAGCGCG
R316Hf: GATCATGTCCCTTCACGCTGCTGTGCGC
R316Hr: GCGCACAGCAGCGTGAAGGGACATGATC

Prokaryotic expression vectors pET15b-TRα, pET15b-TRβ, and mutants pET15b-TRβ(R320C), pET15b-TRβ(R320H), pET15b-TRβ(R316H) were generated by subcloning of the wild-type receptors TRα, TRβ, and mutants cDNAs into the *Nde*I and *Bam*HI sites of pET15b vector using following cloning primers:

TRα *Nde*If:GGGAATTCCATATGGAACAGAAGCCAAGCAAGGTGG
TRα *Bam*HIr: CGCGGATCCTTAGACTTCCTGATCCTCAAAGACC
TRβ *Nde*If: GGAATTCCATATGACCCCCAACAGTATGACAGAAAATGGC
TRβ *Bam*HIr: CGCGGATCCCTAATCCTCGAACACTTCCAGGAACAAAGG

The identity of all constructs was confirmed by restriction enzyme mapping and DNA sequence analysis.

5.4. Cell culture, transient transfection, and luciferase assay

HEK293 cells were seeded at a density of 45,000 cells per well in 24-well culture plate and grown in Dulbecco's modified Eagle's medium with supplemented with 10% fetal bovine serum (MediaTech) 24 h prior to transfection. All cells were routinely maintained on plastic tissue culture plates at 37 °C in a humidified 5% CO_2 containing atmosphere.

Transfections were performed by calcium phosphate coprecipitate method. Triplicate plates of cells were transfected with 140 ng of luciferase reporter plasmid TRE-Luc (DR4-Luc+), 27 ng of control plasmid pRL-CMV, and 80 ng of wild-type or mutant receptor expression vector. Six hours after the transfection, the media was removed and replaced with DMEM + 10% charcoal-resin stripped FBS containing appropriate concentrations of ligand. The cells were allowed to incubate with the new media for 36 h before harvesting by passive lysis.

Luciferase assays were performed by dual luciferase reporter assay (Promega) using an I450 Microbeta luminescence Counter (Perkin–Elmer life Science, Boston, MA). Cells were washed in 1 ml of PBS buffer, followed by lysis in 100 μl 1 × lysis buffer. Cell lysate (20 μl) was immediately transferred into a 96-well Microliter plate 2 (Dynex). The activity of renilla luciferase in the lysate was measured using 100 μl 'Stop & Glo' substrate per sample. Activity is reported as relative light units (RLU) determined as the ratio of the firefly luminescence divided by the luminescence of the renilla luciferase control. The RLU values are normalized such that the maximum inducible expression by T3 with TRβ(wt) or TRα(wt) is arbitrarily set to 100 RLU. Dose–response data were analyzed by nonlinear regression analysis with Graphpad Prism.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.bmc.2005.03.040.

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