Mutant-Selective Thyromimetics for the Chemical Rescue of Thyroid Hormone Receptor Mutants Associated with Resistance to Thyroid Hormone†

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ABSTRACT: The thyroid hormone receptors (TRs) are ligand-dependent transcription factors that control the expression of multiple genes involved in development and homeostasis in response to thyroid hormone (triiodothyronine, T3). Mutations to TRβ that reduce or abolish ligand-dependent transactivation function are associated with resistance to thyroid hormone (RTH), an autosomal dominant human genetic disease. A series of neutral alcohol-based compounds, based on the halogen-free thyromimetic GC-1, have been designed, synthesized, and evaluated in cell-based assays for their ability to selectively rescue three of the most common RTH-associated mutations (i.e., Arg320 → Cys, Arg320 → His, and Arg316 → His) that affect the basic carboxylate-binding arginine cluster of TRβ. Several analogues show improved potency and activity in the mutant receptors relative to the parent compound GC-1. Most significantly, two of these mutant-complementing thyromimics show high potency and activity with a strong preference for the mutant receptors over wild-type TRα(wt), that is associated with the cardiotoxic actions of T3. The compounds were evaluated in reporter gene assays using the four common thyroid hormone response elements, DR4, PAL, F2 (LAP), and TSH, and show activities and selectivites consistent with their unique potential as agents to selectively rescue thyroid function to these RTH-associated mutants.

Thyroid hormone action is mediated by thyroid hormone receptors (TRs),[1] which belong to the superfamily of nuclear/steroid hormone receptors (*1*−*4*). Thyroid hormone receptors function as ligand-dependent transcriptional regulators that control the expression of a specific set of genes involved in development and homeostasis in response to triiodothyronine (T3). Thyroid hormone affects brain development, skeletal maturation, thermogenesis, heart contractility, and the secretion and metabolic turnover of different hormones (*5*, *6*).

TRs bind as homo- or heterodimers to DNA at specific hormone response elements (HREs) found in the promoters of hormone responsive genes. There are two subtypes of the thyroid hormone receptors, TRα and TRβ. Each subtype is expressed in at least two isoforms, TRα1, TRα2, TRβ1, and TRβ2. The isoforms TRα1, TRβ1, and TRβ2 bind thyroid hormone and act as ligand-regulated transcription factors. However, the TRα2 isoform, which is prevalent in the pituitary and other parts of the central nervous system, does not bind thyroid hormones and acts in many contexts as a transcriptional repressor (*2*). Although TRα1, TRβ1, and TRβ2 are structurally and functionally related, they have distinct functional roles. TRα1 has been found in high concentration in skeletal muscle and brain and is closely linked to cardiac function, whereas TRβ is undetectable in kidney and heart tissues. In peripheral tissues, TRβ primarily activates expression of specific genes containing positive thyroid hormone response elements (TREs) in a T3-dependent manner. In the pituitary, TRβ (and not TRα) represses TSH (thyrotropin) expression through a unique negative TRE. TSH stimulates thyroid hormone synthesis in the thyroid. Therefore, TRβ also serves as a critical regulator of the hypothalamic−pituitary−thyroid axis activating genes in peripheral tissues while providing negative feedback regulation of T3 synthesis (*5*, *7*−*10*).

Resistance to thyroid hormone (RTH) is a syndrome characterized by a variable tissue hyposensitivity to thyroid hormone and is primarily caused by autosomal dominant mutations to TRβ (*6*, *11*, *12*). The syndromes of resistance to thyroid hormone are characterized by the clinical and biochemical manifestation of reduced thyroid hormone action relative to circulating hormone levels. Many RTH-associated mutations to TRβs are known to impair or abolish ligand-dependent transactivation function which can lead to a range of clinical presentations such as goiter, learning disabilities, impaired bone maturation, and mental retardation (*6*, *11*, *13*−*17*). Similar (and in some cases identical) somatic mutations of TRβ have been found associated with papillary thyroid cancer and pituitary cancer (TSHoma) (*18*, *19*). Although many mutant receptors show only reduced activity toward T3, clinical treatment of RTH with supraphysiological concentrations of T3 to recover TRβ activity can lead to overstimulation of TRα that has been implicated with undesirable side effects such as tachycardia and heart arrhythmia. This suggests that hormone analogues that can selectively activate mutant TRβ over TRα(wt) may have

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[1] Abbreviations: RTH, resistance to thyroid hormone; TR, thyroid hormone receptor; HRE, hormone response element; TRE, thyroid hormone response element; T3, triiodothyronine; TSH, thyrotropin; RLU, relative light units.

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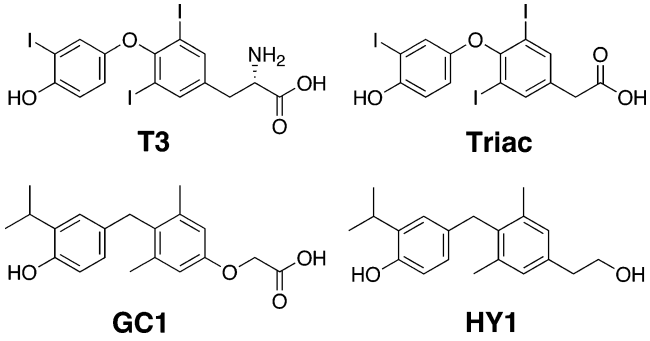


FIGURE 1: Triiodothyronine (T3) and synthetic thyroid hormone analogues.

promise as agents to rescue the dominant negative actions of RTH without leading to cardiotoxic side effects associated with overstimulation of TRα.

We previously reported a neutral alcohol HY1 as a potent mutant-selective agonist for the RTH-associated mutant TRβ-(R320C) (Figure 1). This RTH-complementing analogue was designed on the basis of the T3/TRα crystal structure and represents perhaps the first example of a compound that was designed to complement a mutation associated with a genetic disease (*20*). More recent examples of the chemical rescue of disease-associated mutations involving nuclear receptors include the discovery and development of agonists for mutant forms of the vitamin D receptor associated with rickets (*21*−*23*). Herein we report on the development of a series of alcohol-based analogues of HY1 and the evaluation of their in vitro activities with TRβ(R320C) and related TRβ mutations of a similar "chemical class" (Arg320His and Arg316His) that represent some of the most commonly occurring mutations associated with RTH. As RTH-associated mutants have been observed to affect TR activity differently on different TREs, analogues were evaluated for their ability to selectively activate mutant TRs on the three common positive TREs as well as the negative TSH promotor.

MATERIALS AND METHODS

Radiolabeled L-3,5,3′-[^{125}I]triiodothyronine ([^{125}I]-T3), 799 Ci/mmol, was obtained from PerkinElmer LifeSciences, Inc. (Boston, MA). Unlabeled T3, isopropyl D-thiogalactopyranoside (IPTG), leupeptin, aprotinin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), and yeast extract were from Sigma (St. Louis, MO). The pET15b vector and the competent BL21(DE3)pLysS *Escherichia coli* cells were obtained from Novagen (Madison, WI). Cell culture media was purchased from Mediatech, Inc. (Herndon,VA). Human embryonic kidney 293 (HEK293) cells were maintained at the University of Delaware Cell Culture Core Facility. Transactivation response assays were performed using the dual-luciferase reporter assay system (Promega no. E1960) following the manufacturer's protocol. The Immobilon-NC transfer membrane containing nitrocellulose (0.45 μm) was obtained from Millipore Corp. (Bedford, MA). TRE-PAL×3Luc, TRE-F2×3Luc, and TSHα-Luc plasmids were kindly provided by Dr. S. Refetoff and Dr. L. J. DeGroot (University of Chicago, Chicago, IL) (*24*, *25*).

Molecular Modeling. Molecular modeling was performed on a silicon graphics Octane using Flo98/QXP modeling software (available from Thistlesoft, P.O. Box 227, Colebrook, CT 06021; cmcma@ix.netcom.com) (*26*). Site models representing all residues within 12 Å of TRβ-T3 and TRβ-GC-1 were constructed from published X-ray coordinates of Fletterick et al. (*27*−*29*). The side chains of all residues that contacted the ligand were "free" during all simulations. Site models for the mutant receptors TRβ(R320C) and TRβ-(R320H) were generated by manually changing the side chain of Arg320 and performing 10 rounds of 3 ps simulated annealing with GC-1 bound. Ligands were evaluated using the Monte Carlo docking (mcdock) algorithm of QXP (1000 steps) which minimizes the energy of the ligand-bound complex starting from random conformations and orientations of the ligand. All calculations were performed in the absence of explicit solvent using a distance-dependent dielectric (4.0*r*, Å).

*4-Bromo-2-isopropylphenyl Methoxymethyl Ether (**1**).* To a well-stirred solution of 4-bromo-2-isopropylphenol (21.4 g, 100 mmol) and dimethoxymethane (10 mL, 250 mmol) in 100 mL of methylene chloride was added 10 g of phosphous pentoxide in portion. After the starting material disappeared, the reaction mixture was filtered, and the solid was washed with CH_2Cl_2 (2 × 20 mL). The combined organic layer was washed with 1 N NaOH solution, water, and brine. The organic solution was dried over $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography (silica gel, 95:5 hexane:ethyl acetate) to afford the desired product (24.0 g, 93 mmol, 93%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.2 (d, 6H, J = 6.9 Hz), 3.29 (heptet, 1H, J = 6.9 Hz), 3.47 (s, 3H), 5.17 (s, 2H), 6.94 (d, 1H, J = 8.7 Hz), 7.22 (dd, 1H, J = 8.7, 2.7 Hz), 7.30 (d, 1H, J = 2.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 22.6, 26.9, 56.03, 94.5, 114.4, 115.7, 129.2, 139.9, 153.4. HR-MS: calcd for $C_{11}H_{15}O_2Br$, 258.0255; found, 258.0253.

*2-Bromo-5-(1-ethoxyethoxy)-1,3-dimethylbenzene (**2**).* A solution of 4-bromo-3,5-dimethylphenol (10.0 g, 50 mmol) and *p*-toluenesulfonic acid (TsOH, 200 mg, 1 mmol) in ether (100 mL) was cooled to 0 °C in ice−water bath. A solution of ethyl vinyl ether (5.0 mL, 100 mmol) in 5 mL of ether at 0 °C was dried over molecular sieves (4 Å) for 15 min and then transferred to the phenol solution via cannula. The reaction mixture was stirred at 0 °C for 8 h and then poured into a vigorously stirring solution of saturated aqueous $NaHCO_3$ at 0 °C. The aqueous phase was extracted with 2 × 50 mL of ether. The combined organic phase was washed with 1 N NaOH, water, and brine and then dried over $MgSO_4$. Evaporation of the solvent gave only the protected product, 12.5 g (47.5 mmol, yield 95%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.73 (s, 2H), 5.31 (q, J = 5.3 Hz, 1H), 3.75 (m, 1H), 3.50 (m, 1H), 2.35 (s, 6H), 1.45 (d, J = 5.3 Hz, 3H), 1.17 (t, J = 5.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.40, 20.44, 24.25. 61.57, 99.76, 17.38, 117.38, 119.70, 139.37, 155.62. HRMS: calcd for $C_{12}H_{17}BrO_2$, 272.0412; found, 272.0407.

*4-(1-Ethoxyethoxy)-2,6-dimethylbenzaldehyde (**4**).* To a solution of **2** (12.5 g, 47.5 mmol) in 100 mL of tetrahydrofuran at −78 °C was slowly added 50 mL of *n*-butyllithium (2.5 M in pentane). The reaction mixture was stirred for 30 min at −78 °C before DMF (9 mL, 100 mmol) was added. The reaction mixture was stirred for 30 min at −78 °C and for 1 h at room temperature, then was diluted with 100 mL of ether, washed with 100 mL of water, acidified with 1 N HCl, and washed with 4 × 50 mL of brine. The organic

layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, 90:10 hexane:ethyl acetate) to yield **4** (9.5 g, 35 mmol, 75%) as an oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.46 (s, 1H), 6.66 (s, 2H), 5.47 (q, J = 5.3 Hz, 1H), 3.73 (m, 1H), 3.51 (m, 1H), 2.57 (s, 6H), 1.50 (d, J = 5.3 Hz, 3H), 1.18 (t, J = 5.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.14, 20.04, 21.07, 61.35, 98.75, 117.21, 144.37, 160.30, 191.76. HRMS: calcd for $C_{13}H_{18}O_3$, 222.1256; found, 222.1252.

*[4-(1-Ethoxyethoxy)-2,6-dimethylphenyl](3-isopropyl-4-methoxymethoxyphenyl)methanol (**5**).* To a solution of **1** (10 g, 38 mmol) in 100 mL of tetrahydrofuran at −78 °C was added 29.0 mL of *n*-butyllithium (2.0 M in pentane), and the reaction mixture was stirred for 30 min at −78 °C under argon. A solution of the aldehyde **4** (11.8 g, 39 mmol) in THF anhydrous (100 mL) was added dropwise. The solution was maintained at −78 °C for 1 h and then was stirred for 6 h at room temperature. The reaction mixture was diluted with 150 mL of ether and washed with 200 mL of water and 5 × 50 mL of brine. The combined extracts were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 90:10 hexane:ethyl acetate) to yield **5** (12 g, 24 mmol, 68%) as an oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24 (s, 1H), 6.92 (d, J = 8.5 Hz, 1H), 6.86 (d, J = 0.86 Hz, 1H), 6.60 (s, 2H), 6.22 (d, J = 4.2 Hz, 1H), 5.36 (m, 1H), 5.15 (s, 2H), 3.77 (m, 1H), 3.52 (m, 1H), 3.46 (s, 3H), 3.28 (m, 1H), 2.22 (s, 6H), 1.48 (d, J = 5.3 Hz, 3H), 1.17 (m, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.17, 15.18, 20.40, 20.94, 21.04, 22.74, 27.08, 28.78, 35.61, 55.99, 61.51, 70.84, 94.53, 101.37, 113.55, 123.68, 123.78, 133.03, 136.11, 137.24, 138.61, 153.08, 155.88. HRMS: calcd for $C_{24}H_{34}O_5$, 402.2406; found, 402.2401.

*Trifluoromethanesulfonic Acid 4-(3-Isopropyl-4-methoxymethoxybenzyl)-3,5-dimethylphenyl Ester (**7**).* To a solution of **6** (5.0 g, 15 mmol) in 50 mL of DMF was added K_2CO_3 (4.2, 30 mmol). The mixture was stirred 10 min at room temperature before *p*-nitrophenyl triflate (4.50 g, 16.5 mmol) was added to the reaction mixture. 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 in vacuo to give 6.5 g (90%) of **7** a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (s, 2H), 6.88 (dd, J = 3.2 Hz, 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, $CDCl_3$) δ 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.

*4-(4-[2-(2-Bromoethoxy)tetrahydro-2H-pyran]-2,6-dimethylbenzyl)-2-isopropylphenol (**8**).* To a solution of cesium carbonate (2.26 g, 6.95 mmol) and 4-(4-hydroxy-2,6-dimethylbenzyl)-2-isopropylphenol (*30*) (356 mg, 1.398 mmol) in 20 mL of 37% (v/v) DMF in THF at −25 °C was added 290 mg of 2-(2-bromoethoxy)tetrahydro-2*H*-pyran (1.39 mmol). The reaction mixture was allowed to warm to ambient temperature and was stirred overnight. The reaction mixture was then poured into 20 mL of cold 1 N HCl and extracted with EtOAc (3 × 15 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated under reduced pressure. Silica flash chromatography (10% EtOAc/hexane) afforded 234 mg (44%) of the desired product **8** as a colorless oil (R_f = 0.42, 30% EtOAc/hexane): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.94 (d, J = 2.08 Hz, 1H), 6.68 (d, J = 8.36 Hz, 1H), 6.62 (dd, J = 2.20, 8.28 Hz, 1H), 6.54 (s, 2H), 5.07 (s, 1H), 4.75 (t, J = 3.56 Hz, 1H), 4.0−4.1 (m, 3H), 3.84−3.95 (m, 3H), 3.48−3.57 (m, 1H), 3.21−3.33 (m, 1H), 3.15 (m, 1H), 2.17 (s, 6H), 1.44−1.88 (m, 6H), 1.17 (d, J = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.40, 138.86, 137.18, 132.41, 129.81, 126.24, 125.31, 114.96, 111.71, 99.07, 67.86, 66.19, 62.11, 33.88, 30.73, 27.34, 25.68, 22.80, 20.52, 19.36. CIMS: calcd for $C_{25}H_{34}O_4$, 398; found, 398.

*4-[4-(2-Hydroxyethoxy)-2,6-dimethylbenzyl]-2-isopropylphenol (**KG-2**).* To a solution of **8** (30 mg, 0.075 mmol) in 5 mL of MeOH was added toluenesulfonic acid (5 mg). The solution was stirred at ambient temperature overnight, the solvent was removed under reduced pressure, and the residue was purified by silica flash chromatography (10% EtOAc/hexane) to afford 23.27 mg (99%) of the desired alcohol as a white solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.96 (d, J = 1.9 Hz, 1H), 6.70−6.62 (m, 2H), 6.55 (s, 2H), 4.58 (br s, 1H), 4.03 (t, J = 3.4 Hz, 2H), 3.97 (m, 2H), 3.90 (s, 2H), 3.27 (septet, J = 6.9, 1H), 2.18 (s, 6H), 1.57 (br s, 1H), 1.17 (d, J = 6.9 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.85, 153.61, 138.88, 137.09, 132.81, 129.85, 126.30, 125.41, 114.94, 111.78, 69.66, 62.00, 33.84, 27.17, 22.96, 20.50. HRMS: calcd for $C_{20}H_{26}O_3$, 314.1882; found, 314.1882.

*4-[4-(3-Isopropyl-4-methoxymethoxybenzyl)-3,5-dimethylphenoxy]-but-2-yn-1-ol (**9**).* A solution of **5** (126 mg, 0.4 mmol) in 5 mL of DMF was added Cs_2CO_3 (370 mg, 1.1 mmol) and stirred 10 min at room temperature; then 4-bromobut-2-yn-1-ol (150 mg, 1 mmol) was added to the reaction mixture and stirred at room temperature until the starting material disappeared. Water (20 mL) and ether (10 mL) were added, and the aqueous layer was separated. The organic extract was washed with water (20 mL) and brine and dried over $MgSO_4$. The solvent was evaporated in vacuo, and the residue was purified by chromatography (hexane/ethyl acetate 90/10) to give **9**, 105 mg (70%), as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (s, 1H), 6.88 (d, J = 8.4 Hz, 1H), 6.65 (s, 2H), 6.60 (dd, J = 2.0 Hz, 1H), 5.13 (s, 2H), 4.69 (t, J = 2.4 Hz, 2H), 4.31 (t, J = 1.8 Hz, 2H), 3.90 (s, 2H), 3.45 (s, 3H), 3.27 (m, 1H), 2.20 (s, 6H), 1.18 (d, J = 8.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.77, 152.70, 138.68, 133.29, 130.75, 126.22, 125.51, 114.38, 114.10, 94.81, 85.42, 81.28, 56.20, 56.08, 51.42, 34.00, 29.92, 27.17, 23.01, 20.79. HRMS: calcd for $C_{24}H_{30}O_4$, 382.2144; found, 382.2144.

*4-[4-(4-Hydroxybut-2-ynyloxy)-2,6-dimethylbenzyl]-2-isopropylphenol (**KG-8**).* To a solution of **9** (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 **KG-8**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.91 (s, 1H), 6.65 (s, 2H), 6.56 (dd, J = 8.4 Hz, 2H), 4.69 (t, J = 1.6 Hz, 2H), 4.56 (br

s, 1H), 4.31 (t, $J = 1.8$ Hz, 2H), 3.88 (s, 2H), 3.13 (m, 1H), 2.20 (s, 6H), 1.20 (d, $J = 8.0$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.78, 150.92, 138.70, 130.80, 126.39, 125.56, 115.33, 114.39, 85.44, 81.30, 56.10, 51.44, 33.95, 27.32, 22.77, 20.77. HRMS: calcd for $C_{22}H_{26}O_3$, 338.1882; found, 338.1882.

*3-[4-(3-Isopropyl-4-methoxymethoxybenzyl)-3,5-dimethylphenyl]-prop-2-yn-1-ol (**10**).* A solution of 100 mg (0.3 mmol) of **7**, 20 mg (0.1 mmol) of copper(I) iodide (20 mg, 0.1 mmol), tetrabutylammonium iodide (370 mg, 1 mmol), and 0.4 mL of triethylamine in 2 mL of DMF was sparged with nitrogen for 10 min before 25 mg (0.03 mmol) of bis(triphenylphosphine)−palladium dichloride was added. After the mixture was stirred at room temperature for 10 min, 0.1 mL (1.5 mmol) of propargyl alcohol was added, and the reaction mixture was heated at 70 °C for 4 h. The reaction was then cooled to room temperature, diluted with methylene chloride (20 mL), washed with 1 N HCl (10 mL), water (2 × 20 mL), and brine (20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give 90 mg (0.25 mmol, 85%) of **10**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 (s, 2H), 6.88 (dd, $J = 3.8$ Hz, 2H), 6.59 (d, $J = 8.4$ Hz, 1H), 5.13 (s, 2H), 4.48 (d, $J = 4.0$ Hz, 2H), 3.94 (s, 2H), 3.45 (s, 3H), 3.26 (m, 1H), 2.20 (s, 6H), 1.16 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.79, 138.57, 137.69, 137.52, 132.33, 131.41, 126.16, 125.54, 120.11, 114.12, 94.77, 86.54, 56.21, 51.96, 34.57, 31.15, 22.98, 20.27. HRMS: calcd for $C_{23}H_{26}O_3$, 352.2039; found, 352.2038.

*3,5-Dimethyl-4-(3′-isopropyl-4′-methoxybenzyl)benzoic Acid (**12**).* To a rapidly stirring solution of 4-(4-vinyl-2,6-dimethylbenzyl)-2-isopropylphenol (**11**) (*20*) (147 mg, 0.5 mmol) in 5 mL of 30% (v/v) *tert*-butyl alcohol/water at 0 °C was added dropwise a solution of 213 mg (1.35 mmol) of $KMnO_4$ in 2 mL of H_2O. The pH of the reaction mixture was maintained slightly basic by the addition of Na_2CO_3. The reaction mixture was filtered, and the filter cake was washed with H_2O. The combined filtrates were acidified and extracted with ether. The combined organic extracts were washed with H_2O and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash silica chromatography, 2% MeOH/CH_2Cl_2, to afford the desired carboxylic acid as a white solid (110 mg, 71%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83 (s, 2H), 6.95 (d, $J = 1.9$ Hz, 1H), 6.70 (d, $J = 8.4$ Hz, 1H), 6.53 (dd, $J = 1.9$, 8.4 Hz, 1H), 4.05 (s, 2H), 3.79 (s, 3H), 3.28 (q, $J = 6.90$ Hz, 1H), 2.33 (s, 6H), 1.18 (d, $J = 6.9$ Hz, 6H); ^{13}C NMR (100 MHz) δ 172.59, 155.39, 144.42, 137.88, 137.29, 130.43, 130.00, 127.09, 126.23, 125.38, 110.63, 55.66, 34.92, 27.04, 22.91, 20.47.

*3,5-Dimethyl-4-(3′-isopropyl-4′-hydroxybenzyl)benzoic Acid (**13**).* A solution of 28 mg (0.132 mmol) of 3,5-dimethyl-4-(3′-isopropyl-4′-methoxybenzyl)benzoic acid in 1 mL of HOAc and 1 mL of HI(aq) was heated to reflux for 7 h. The solution was reduced to 0.5 mL under reduced pressure before being extracted with CH_2Cl_2 (3 × 4 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (CH_2Cl_2/MeOH, 95/5) to afford 12.4 mg (31%) of the desired phenol: ^{1}H NMR (400 MHz, CD_3OD) δ 7.62 (s, 2H), 6.73 (d, $J = 1.9$ Hz, 1H), 6.52 (d, $J = 8.2$ Hz, 1H), 6.43 (dd, $J = 8.2$, 2.1 Hz), 3.92 (s, 2H), 3.11 (septet, $J = 6.8$ Hz, 1H), 2.20 (s, 6H), 1.03 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 171.9, 153.5, 144.9, 138.7, 136.2, 130.7, 130.5, 126.9, 126.5, 116.1, 35.5, 28.2, 23.2, 20.5.

*4-[4-(Hydroxymethyl)-2,6-dimethylbenzyl]-2-isopropylphenol (**KG-1**).* To a solution of 3,5-dimethyl-4-(3′-isopropyl-4′-hydroxybenzyl)benzoic acid (**13**) (40 mg, 0.134 mmol) in 10 mL of dry ether was added $LiAlH_4$ (6.38 mg, 0.163 mg). The reaction mixture was heated to reflux for 16 h and cooled to ambient temperature before 0.1 mL of EtOAc and 0.05 mL of H_2O were added. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica flash chromatography (hexane/EtOAc, 90/10) to afford 32.5 mg of the desired alcohol (85.4%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 2H), 6.94 (d, $J = 1.74$ Hz, 1H), 6.83 (d, $J = 8.3$ Hz, 1H), 6.62 (d, $J = 8.3$ Hz, 1H), 4.04 (s, 2H), 3.79 (s, 2H), 3.28 (septet, $J = 3.7$ Hz, 1H), 2.31 (s, 6H), 1.16 (d, $J = 6.92$ Hz, 6H); ^{13}C NMR (100 MHz) δ 155.3, 144.3, 137.8, 137.2, 136.8, 130.3, 129.9, 126.0, 125.1, 110.4, 55.6, 27.1, 23.7, 22.8, 20.7. HRMS: calcd for $C_{21}H_{24}O_2$, 308.1776; found, 308.1776.

*4-[4-(3-Hydroxyprop-1-ynyl)-2,6-dimethylbenzyl]-2-isopropylphenol (**KG-9**).* MOM deprotection of **10** was carried out following the same procedure reported for **KG-8** (40 mg, 56%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (s, 2H), 6.88 (s, 1H), 6.58 (d, $J = 8.0$ Hz, 2H), 6.51 (d, $J = 8.0$ Hz, 1H), 4.48 (d, $J = 4.0$ Hz, 2H), 3.94 (s, 2H), 3.15 (m, 1H), 2.19 (s, 6H), 1.18 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.23, 138.67, 137.52, 134.60, 131.39, 131.23, 126.31, 125.52, 120.07, 115.36, 86.52, 86.25, 58.73, 51.90, 34.50, 27.24, 22.74, 20.24. HRMS: calcd for $C_{19}H_{25}O_2$, 284.1854; found, 284.1861.

*Methyl [4-(3-Isopropyl-4-methoxybenzyl)-3,5-dimethylphenyl]-2-propenoate (**15**).* A mixture of 3,5-dimethyl-4-(3′-isopropyl-4′-methoxybenzyl)phenyltrifluoromethanesulfonate (*20*) (1 g, 2.40 mmol), methyl acrylate (0.66 mL, 7.34 mmol), triphenylphosphine (129 mg, 0.49 mmol), and palladium acetate (110 mg, 0.49 mmol) was heated to reflux for 24 h. The reaction mixture was diluted with 50 mL of CH_2Cl_2, washed with 3 × 40 mL of H_2O, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (hexane/EtOAc, 95/5) to give 430 mg (50.1%) of product as a white solid: $R_f = 0.41$ (hexane/EtOAc, 90/10); mp 55.1−56.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, $J = 16.04$ Hz, 1H), 7.23 (s, 2H), 6.95 (d, $J = 2.08$ Hz, 1H), 6.68 (d, $J = 8.36$ Hz, 1H), 6.62 (dd, $J = 2.20$ Hz, $J = 8.36$ Hz, 1H), 6.42 (d, $J = 16.00$ Hz, 1H), 3.99 (s, 2H), 3.80 (s, 3H), 3.77 (s, 3H), 3.26 (qt, $J = 6.84$ Hz, 1H), 2.26 (s, 6H), 1.16 (d, $J = 6.96$ Hz, 6H); ^{13}C NMR (100 MHz) δ 168.15, 155.46, 145.58, 140.86, 138.19, 137.34, 132.52, 131.09, 131.09, 128.29, 126.42, 125.46, 117.04, 110.72, 55.80, 52.05, 34.89, 27.19, 23.08, 20.66.

*[4-(3-Isopropyl-4-methoxybenzyl)-3,5-dimethylphenyl]-3-propanol (**16**).* To a solution of **15** (30 mg, 0.085 mmol) in dry THF (5 mL) was added lithium aluminum hydride (3.20 mg, 0.085 mmol). The reaction mixture was stirred at room temperature for 12 h, followed by slow addition of ethyl acetate and water. The filtrate was concentrated under reduced pressure, and the residue was purified by flash silica chromatography (hexane/EtOAc, 90/10) to give the alcohol,

27.2 mg (98.2%), as a colorless oil: $R_f = 0.28$ (hexane/EtOAc = 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (s, 2H), 6.82 (d, $J = 1.94$ Hz, 1H), 6.57 (d, $J = 8.16$ Hz, 1H), 6.50 (dd, $J = 2.22$ Hz, $J = 8.18$ Hz, 1H), 3.90 (s, 2H), 3.20 (qt, $J = 6.89$ Hz, 1H), 2.83 (t, $J = 7.59$ Hz, 2H), 2.58 (t, $J = 7.97$ Hz, 2H), 2.58 (s, 6H), 1.12 (d, $J = 7.64$ Hz, 6H); ^{13}C NMR (100 MHz) δ 177.13, 153.56, 139.77, 138.24, 136.81, 135.97, 131.93, 129.10, 126.86, 126.49, 115.97, 37.11, 35.00, 31.83, 28.17, 23.20, 20.44; ESI-MS m/z = 349.3 ($C_{22}H_{30}O_2$·Na).

*4-[4-(3-Hydroxypropyl)-2,6-dimethylbenzyl]-2-isopropylphenol (**KG-3**).* To a solution of alcohol **16** (40 mg, 0.12 mmol) in 10 mL of DMF was added 42 mg (0.6 mmol) of $NaSCH_3$. The mixture was stirred at 150 °C for 3 h, then poured into iced water, acidified with 1 N HCl, and extracted with Et_2O. The combined organic phase was washed with H_2O and dried over $MgSO_4$. After evaporation of solvent, the resulting residue was purified by flash chromatography on silica gel using a solution of hexane/EtOAc (90/10) to afford 26 mg (69.4%) of product as a white solid: mp 96.2−96.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (d, $J = 1.80$ Hz, 1H), 6.89 (s, 2H), 6.58 (d, $J = 8.12$ Hz, 1H), 6.54 (dd, $J = 2.08$ Hz, $J = 8.20$ Hz, 1H), 4.78 (br s, 1H), 3.93 (s, 2H), 3.70 (t, $J = 6.44$ Hz, 2H), 3.14 (qt, $J = 6.94$ Hz, 1H), 2.64 (t, $J = 7.36$ Hz, 2H), 2.21 (s, 6H), 1.94−1.87 (m, 2H), 1.21 (d, $J = 6.96$ Hz, 6H); ^{13}C NMR (100 MHz) δ 150.96, 139.56, 137.31, 135.09, 134.35, 132.22, 128.35, 126.48, 125.54, 115.30, 62.76, 34.44, 34.30, 31.83, 27.29, 22.77, 20.43. HRMS (CI): calcd for $C_{21}H_{28}O_2$, 312.2089; found, 312.2080.

Plasmid Constructions. Plasmids pSG5-TRβ mutants R320C, R320H, and R316H were constructed from the parent plasmid pSG5-TRβ (encoding the TRβ1 isoform) by oligonucleotide-directed mutagenesis using Quickchange (Stratagene) using the following oligonucleotides: R320Cf, CGCGCTGCTGTGTGCTATGACCCGGAAAG; R320Cr, CTTTCCGGGTCATAGCACACAGCAGCGCG; R320Hf, CGCGCTGCTGTGCACTATGACCCGGAAAGTG; R320h, CACTTTCCGGGTCATAGTGCACAGCAGCGCG; R316Hf, GATCATGTCCCTTCACGCTGCTGTGCGC; R316h, GCGCACAGCAGCGTGAAGGGACATGATC.

Prokaryotic expression vectors pET15b-TRα and pET15b-TRβ and mutants pET15b-TRβ (R320C), pET15b-TRβ (R320H), and pET15b-TRβ (R316H) were generated by subcloning of the wild-type receptors TRα1, TRβ1, and mutant cDNAs into the *Nde*I and *Bam*HI sites of pET15b vector using following cloning primers: TRα NdeIf, GGGAATTCCATATGGAACAGAAGCCAAGCAAGGTGG; TRα BamHIr, CGCGGATCCTTAGACTTCCTGATCCTCAAAGACC; TRβ NdeIf, GGAATTCCATATGACCCCCAACAGTATGACAGAAAATGGC; TRβ BamHIr, CGCGGATCCCTAATCCTCGAACACTTCCAGGAACAAAGG. The identity of all constructs was confirmed by restriction enzyme mapping and DNA sequence analysis.

Cell Culture, Transient Transfection, and Luciferase Assays. HEK293 cells were seeded at a density of 45000 cells per well in 24-well culture plates and grown in Dulbecco's modified Eagle's medium 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 the calcium phosphate coprecipitate method. Triplicate wells of cells were transfected with 140 ng/well luciferase reporter plasmid TRE-Luc (DR4-Luc+, PAL×3Luc, F2×3Luc, or TSHα-Luc), 27 ng/well control plasmid pRL-CMV, and 80 ng/well wild-type or mutant receptor expression vector. Six hours after the transfection, the medium was removed and replaced with DMEM plus 10% charcoal-resin-stripped FBS containing appropriate concentrations of ligand. The cells were allowed to incubate with the new media for 36 h before being harvested by passive lysis.

Luciferase assays were performed by dual luciferase reporter assay (Promega) using an I450 microbeta luminescence counter (PerkinElmer Life Sciences, Boston, MA) following the manufacturer's protocol. 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) is arbitrarily set to 100 RLU.

Binding Assay. The His-tag vector pET15b encoding wild-type or mutant TRs [pET15b-TRα, pET15b-TRβ, mutants pET15b-TRβ (R320C), pET15b-TRβ (R320H), or pET15b-TRβ (R316H)] was expressed in *E. coli* according to the procedure described previously. Briefly, 1 L of 2XYT media was inoculated with overnight culture (5 mL) of *E. coli* BL21(DE3)pLysS cells expressing one of the above plasmids. The culture was grown at 37 °C to an OD_{600nm} of 1.0−1.2, induced with 0.5 mM IPTG, and incubated at 30 °C for 3 h. The cells were harvested by centrifugation (15 min at 6000*g*, 4 °C), and the cell paste was resuspended in 10 mL of cold lysis buffer A [10% (v/v) glycerol, 20 mM Tris-HCl, 500 mM NaCl, 10 mM mercaptoethanol, pH 7.9] with protease inhibitors (5 μg/mL leupeptin, 50 μg/mL aprotinin, 5 μg/mL pepstatin A, and 0.2 mM PMSF). The suspension was sonciated (Branson sonicator with microprobe) for 3 × 10 s at 60% power. The cell debris and the pellet fraction were separated from the supernatant by centrifugation (20 min at 10000*g*, 4 °C). The crude supernatant could be used directly or stored at −80 °C.

The nitrocellulose filter-binding assay described by Inoue et al. (*19*) was with minor modification used for radioligand competition binding assays. Briefly, 1−5 μL of the above crude protein was added into buffer B [10% (v/v) glycerol, 20 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, 5 mM mercaptoethanol, pH 7.6] and incubated at 4 °C for 18 h with 10−40 fmol of [^{125}I]-T3 (10 fmol for wild-type receptors, 40 fmol for mutants) in the absence or presence of various concentrations of synthetic ligands. Nonspecific binding was measured in the presence of a 1000-fold excess of unlabeled T3. The protein-bound fractions were collected onto nitrocellulose membranes, which were exposed to phosphor screen and counted by a PhosphorImager (Molecular Dynamics). Association constants were determined by nonlinear regression analysis using GraphPad Prism. Since, in independent binding assays, the K_a of the WT TRβ varied from 0.2×10^9 to 0.8×10^9, K_a's of the mutant were normalized by dividing their value by that of the TRβ K_a which was determined with each assay.

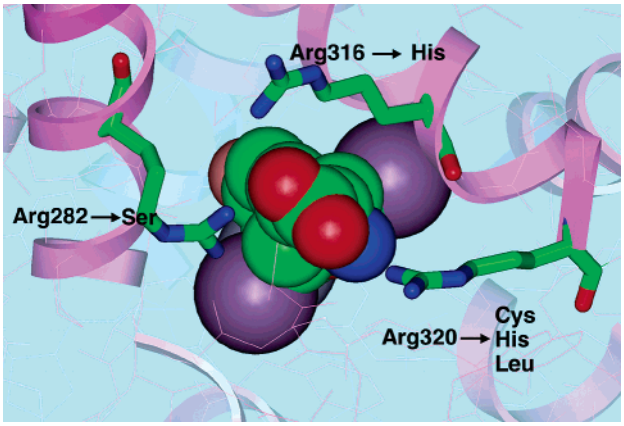


FIGURE 2: Complex of the TRβ/T3 cocrystal structure highlighting the basic carboxylate-binding cluster of arginines (*27*). Common RTH mutations affecting the Arg cluster are shown.

RESULTS

Mutations of the Carboxylate-Binding Arginine Cluster. The TRs contain a cluster of basic residues (TRβ: Arg282, Arg316, Arg320) that form a carboxylate-binding site for the natural hormone T3 (*27*). A similar pattern of basic residues is found in the RARs (retinoic acid receptors) and the RXRs (retinoid-X receptors) which also bind carboxylate-containing hormones (*31*, *32*). The guanidinium groups of the three arginines are within 5 Å of each other and are not paired to any other acid (anionic) residues in the ligand-bound complex. It is therefore intriguing that these three arginines are critical for binding the zwitterionic but overall neutral hormone T3 (Figure 2). Mutations to the arginine cluster are among the most prevalent mutations associated with resistance to thyroid hormone and include R282S, R316H, R320C, R320L, and R320H (*33*). Missense mutations of TRβ to the arginine cluster typically result in reduced responsiveness to T3 and therefore disrupt expression of TRβ-responsive genes in peripheral tissues as well as normal negative feedback regulation along the hypothalamic−pituitary−thyroid axis of which TRβ is a critical regulator.

Design Considerations. Previously, we have shown that the T3-TR cocrystal structure could be used as a guide to design mutant-selective thyromimetics capable of rescuing activity to the RTH-associated mutant, R320C, in preference to the wild-type TR subtypes, TRα and TRβ (*20*). The crystal structures of the ligand-binding domain (LDB) of both TRα and TRβ have been solved and provide the basis for the rational molecular design of synthetic thyromimetics. The cocrystal structures of the T3-bound and GC-1-bound form of TRβ show an encapsulated hormone-binding mode where the ligand is entirely buried within the hydrophobic interior of the ligand-binding pocket (*28*). Polar contacts between the 4′-phenolic hydroxyl and His435 at one end of the ligand, and the side-chain carboxylate with the arginine cluster, define the orientation of the ligand in the receptor. The carboxylate of GC-1 hydrogen bonds directly with Arg320 and Arg316 in addition to a water-mediated hydrogen bond with Arg316.

HY1 is a mutant-selective thyromimetic that is 5 times more potent as an agonist for TRβ(R320C) than the parent thyromimetic GC-1 and is 5 times more selective for the mutant TRβ(R320C) than TRα(wt). Although this TRβ mutant still retains two of three arginines that normally interact with the ligand's carboxylate, the neutral alcohol HY1 partially but, more importantly, selectively restores activity to the mutant. Therefore, much of our efforts have focused on the design and synthesis of neutral alcohol-functionalized analogues for TRβ(R320C) and receptors with related mutations that affect carboxylate binding.

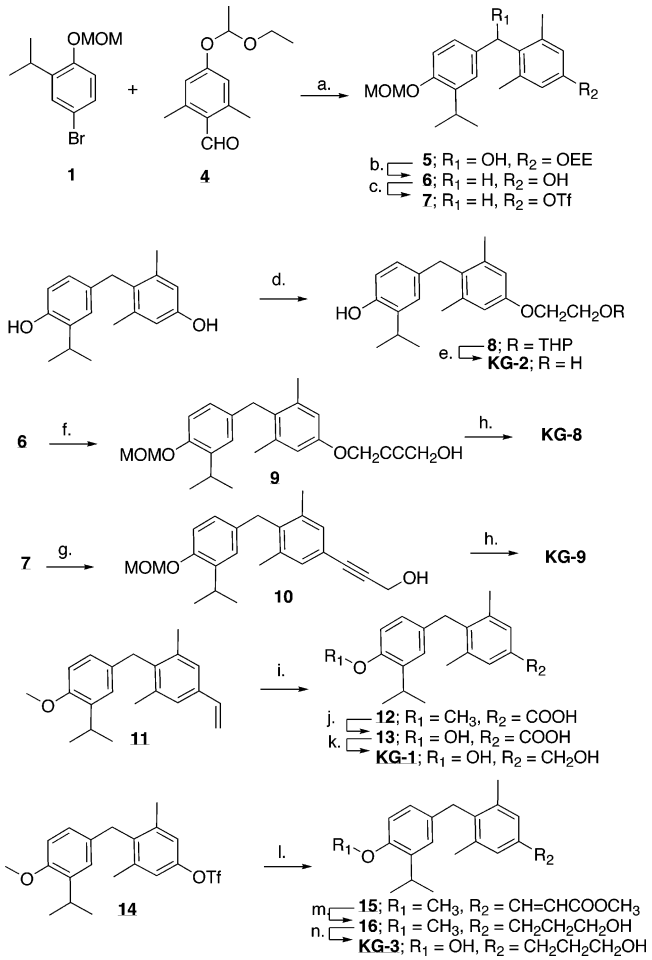
The recently reported crystal structures of mutant forms of TRβ associated with RTH provide one of the first glimpses of the structural changes responsible for a genetic disease (*34*, *35*). The TRβ(R316H)/Triac cocrystal structure reveals that mutation of Arg316, a member of the carboxylate-binding arginine cluster on helix 6 that hydrogen bonds to helix 1, causes significant structural changes to the receptor-binding pocket. While these structural insights help to define the unique challenges associated with designing mutant-complementing analogues for Arg316 mutants of TRβ, our goal is to develop compounds that can induce a receptor conformation similar to that of the active wild-type receptor. Therefore, for these studies docking simulations were performed with site models derived from the coordinates of the agonist-bound forms of the wild-type receptor, which may provide a better basis to design analogues capable of functional rescue.

The availability of cocrystal structures of both T3- and GC-1-bound TRβ(wt) can aid in the design of new mutant-selective thyromimetics for rescuing RTH mutants. Standard methods for structure-based design are limited when applied to the investigation of mutations involving the arginine cluster for several reasons. First, the close proximity of the three guanidiniums of the arginine cluster makes it unlikely that all three residues would be simultaneously protonated. Second, the protonation state of the arginine cluster is likely to change upon ligand binding or with changes in ligand conformation. Although GC-1 and T3 are isosteric, their carboxylates interact differently with the arginine cluster, suggesting that even subtle changes to ligand structure can change the mode of interaction with the arginine cluster. Though limited in their ability to predict potency in these systems, structure-based design still plays an important role in selecting molecules of appropriate size and geometry.

Ligand Design and Synthesis. Based on our previous success with the neutral alcohol HY1, a series of alcohol-based thyromimics, **KG-1**, **KG-2**, **KG-3**, **KG-8**, and **KG-9** were designed to selectively complement the RTH-associated mutants TRβ(R320C) and TRβ(R316H), based on site models constructed from the reported T3- and GC-1-bound cocrystal structure of TRβ (*4*, *27*). **KG-1**, **KG-2**, and **KG-3** were derived from previously described intermediates **11** and **14** (Scheme 1) (*30*, *36*). The previously reported intermediate **6** was synthesized using an alternative strategy which employs the selective reactivity of the ethoxyethyl protecting group in the presence of the methoxymethyl group of intermediate **5**.

Cellular Reporter Gene Activity. The actions of TRs on gene regulation are achieved through their selective binding to specific DNA sequences within the promotor regions of hormone responsive genes, known as "thyroid response elements" (TREs). Both liganded and unliganded forms of TRs can bind to DNA as monomers, homodimers, or heterodimers with auxiliary proteins, such as the retinoid X receptor (RXR). Dimers generally repress transcription of target genes in the absence of bound hormone and activate

Scheme 1: Synthetic Route and Reaction Agents and Conditions[a]



[a] Reagents/conditions: (a) *n*-BuLi, THF, −78 °C to room temperature; (b) 10% Pd/C, H_2, EtOH−AcOH (10:1), room temperature, 12 h; (c) 4-nitrophenyl triflate/K_2CO_3, DMF, room temperature, 4 h; (d) $BrCH_2CH_2OTHP/Cs_2CO_3$, DMF, room temperature, 8 h; (e) TsOH, MeOH; (f) $BrCH_2CCCH_2OH/K_2CO_3$, DMF, room temperature, 8 h; (g) $Pd(PPh_3)_2Cl_2$/propargyl alcohol, TBAI, CuI, DMF, 70 °C, 4 h; (h) HCl, MeOH/H_2O; (i) $KMnO_4$, tBuOH, Na_2CO_3; (j) HOAc, HI; (k) $LiAlH_4$, Et_2O; (l) $Pd(OAc)_2$, PPh_3, methyl acrylate; (m) $LiAlH_4$, THF; (n) $NaSCH_3$, DMF.

transcription when hormone is bound. TREs are composed of two or more AGGTCA-like half-site motifs, arranged in unique orientations and spacings. TREs are arranged in either direct repeats separated by four bases (called DR4), in palindromes (TREpal), or in an inverted palindrome separated by six bases (IP, TRE-lap, or TRE-F2). Monomeric binding is relatively weak and nonspecific compared with dimeric binding (*1*, *2*).

Effects of the Ligands on TRs and Mutants R320H, R320C, and R316H at DR4 Sites. Of the three common TR response elements found in the promoters of TR responsive genes, direct repeats (e.g., DR4) are the most common (*1*, *2*). To test the activity of thyromimetics in mediating thyroid hormone response through the TRα, TR*β*, and RTH-associated TR*β* mutations, DR4 responsive reporter gene assays were performed using HEK293 cells transiently cotransfected with pSG5-TR and the enhanced luciferase reporter DR4-Luc+ vector.

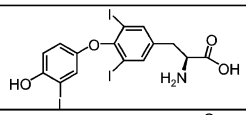
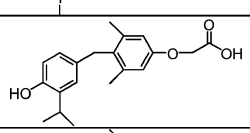
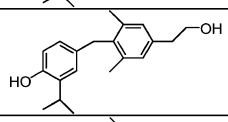
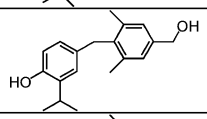
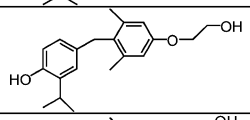
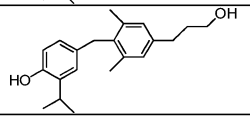
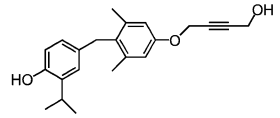
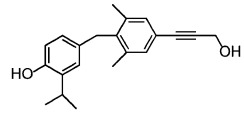
We initially evaluated the dose−response behavior of T3, GC-1, HY1, and the five new analogues for their ability to selectively activate TR*β*(wt) and TRα(wt), as well as the RTH-associated mutants R320H, R320C, and R316H. The potencies (EC_{50}) and activities (percent maximum inducible activity compared to wild type with T3) are summarized in Table 1. The ligand selectivity was determined by the ratio of EC_{50}(wt) to EC_{50}(mutant) for the same ligand. In the absence of transfected TR receptors, little to no T3-inducible reporter gene activity (induction <1.4-fold) was observed, whereas in the presence of transfected TRs or mutant receptors, DR4-Luc+ activity was stimulated 5−6-fold by treatment of T3 or thyromimetics.

The natural hormone, T3, shows reduced potency (higher EC_{50}) with the three mutants TR*β*(R316H), TR*β*(R320C), and TR*β*(R320H). The natural hormone T3 is 10, 6, and 51 times less potent toward the RTH-associated TR*β* mutants R320C, R320H, and R316H than TR*β*(wt) and 29, 17, and 152 times less potent toward these mutants than TRα(wt) (Figure 3A). These findings are consistent with the notion that the supraphysiological concentrations of T3 required to significantly activate these RTH-associated mutants would likely lead to overactivation of TRα. T3 also shows reduced efficacy toward these RTH-associated mutants; the maximum inducible activity is reduced 28−50% compared to TR*β*-(wt). The low potency and activity observed with R316H (EC_{50} = 22.8 nM, 50% efficacy) are not surprising given that the recently reported crystal structure of this mutant receptor cocrystallized with Triac shows distortions to the normal structure of the receptor's ligand-binding domain (*35*). A priori, it is not obvious if any ligand can improve both potency and efficacy for these mutants.

The thyromimetic GC-1 offers a convenient halogen-free scaffold from which our mutant-complementing analogues are based. GC-1 is 10, 5, and 27 times less potent in the TR*β* mutants R320C, R320H, and R316H than in TR*β*(wt). Although GC-1 has notable selectivity for TR*β*(wt) over TRα(wt), GC-1 is still 6-, 3-, and 14-fold less potent and 18−50% less active with the TR*β* mutants R320C, R320H, and R316C than with TRα(wt) (Figure 3B).

The cellular dose−response behaviors of HY1 and five new analogues of HY1 were evaluated for their ability to selectively activate the RTH-associated mutants TR*β*-(R320H), TR*β*(R320C), and TR*β*(R316H) in preference to hTR*β*(wt) and hTRα(wt) (Figure 4). Previously, we reported that HY1 was a selective agonist for the RTH-associated mutant TR*β*(R320C). HY1 is 5 times more potent than the parent compound GC-1 toward this mutant. Although HY1 (EC_{50} = 7.0 nM) is not as potent as T3 toward TR*β*(R320C), it has the opposite selectivity, being 5-fold more selective for the mutant than for TR*β*(wt) and 5-fold more selective than for TRα(wt). Furthermore, although HY1 is not a full agonist toward TR*β*(R320C), it still retains significant efficacy (71%). Taken together, the dose−response behavior or HY1 clearly supports the notion that it may have the ability to release the dominant negative actions of the mutant allele without overstimulating the wild-type TR*β* or the TRα subtype which is associated with potential cardiotoxic side effects. In contrast, HY1 is almost a full agonist with TR*β*-(R320H), (maximal activity = 96%) but has nominal selectivity (1.3-fold) compared to the TRα(wt). These results are nonetheless significant because they suggest that the appropriate ligand structure can restore receptor activity as well as potency.

Table 1: Potencies and Efficacies of Natural and Synthetic Ligands for TRα, TR*β*, TR*β*(R320C), and TR*β*(R320H) on DR4-Driven Reporter Gene Expression[a]

Ligand	Structure	**TRα** EC_{50} nM (% max)	**TRβ** EC_{50} nM (% max)	**R316H** EC_{50} nM (% max)	α mut	β mut	**R320C** EC_{50} nM (% max)	α mut	β mut	**R320H** EC_{50} nM (% max)	α mut	β mut
T3		**0.15±0.02** (110)	**0.45±0.10** (100)	**22.8±3.0** (50)	1/ 152	1/ 51	**4.3±0.5** (72)	1/ 29	1/ 10	**2.5±0.3** (70)	1/ 17	1/ 6
GC-1		**6.6±1.1** (100)	**3.6±1.0** (100)	**96.7±19** (50)	1/ 14	1/ 27	**37.7±10.8** (72)	1/ 6	1/ 10	**19.0±4.0** (82)	1/ 3	1/ 5
HY1		**37.7±8.0** (100)	**32.1±6.7** (100)	**42.6±8.8** (56)	1/ 1	1/ 1	**7.0±2.0** (71)	5.3	4.5	**28.9±6.0** (96)	1.3	1.1
KG-1		**278±60** (42)	**314±60** (39)	**47.7±10** (36)	5.8	6.6	**60.7±12.3** (30)	4.6	5.2	**70.1±1.5** (31)	3.9	4.5
KG-2		**327±60** (100)	**223±50** (100)	**888±200** (76)	1/ 3	1/ 4	**131±25.6** (61)	2.4	1.7	**536±110** (100)	1/ 2	1/ 2
KG-3		**28.0±5.0** (98)	**5.3±1.0** (100)	**252±50** (56)	1/ 9	1/ 47	**20.5±5.0** (70)	1.4	1/ 3.8	**30.5±6.2** (68)	1/ 1	1/ 6
KG-8		**33.5±3.5** (100)	**15.6±1.6** (100)	**25.8±3.0** (98)	1.3	1/ 2	**2.7±0.30** (90)	12	5.7	**7.5±0.80** (100)	4.5	2.1
KG-9		**17.2±2.0** (100)	**27.5±3.0** (100)	**21.3±2.8** (94)	1/ 1.2	1.3	**21.2±3.0** (83)	1/ 1.2	1.3	**19.2±2.1** (100)	1/ 1.1	1.4

[a] Efficacies reported in parentheses as percentage of the maximum inducible activity observed with TR*β*(wt) and T3. Mutant to wild-type selectivities, denoted as α/mut, are defined as the ratio [EC_{50} with TRα(wt)]/(EC_{50} with mutant TR*β*).

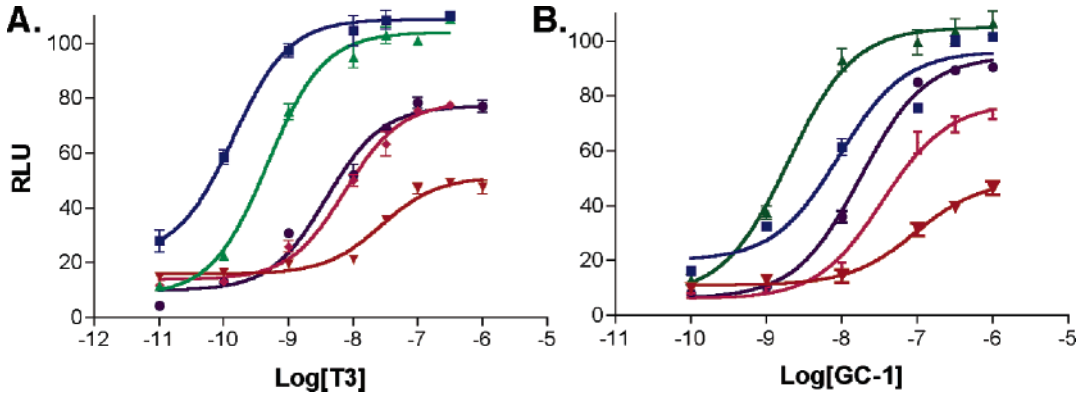


FIGURE 3: Cellular dose−response behavior of (A) T3 and (B) GC-1 with TRα(wt) (blue ■), TR*β*(wt) (green ▲), and RTH-associated mutants TR*β*(R320C) (rose ◆), TR*β*(R320H) (magenta ●), and R*β*(R316H) (red ▼) toward a DR4-luciferase reporter shows reduced potency and activity toward mutant receptors compared to wild type. RLU = relative light units; maximum inducible activity with TR*β*(wt) and T3 was set to 100 RLU.

Analogues **KG-1** and **KG-3**, which shorten or lengthen the alcohol side chain of HY1 by one methylene unit, are active but show reduced potency toward TR*β*(R320C), suggesting that HY1 has the optimal length for interacting with this mutant. Substitution of the phenyl ether oxygen of GC-1 with a methylene has been shown to increase the potency (lower EC_{50}) of GC-1 analogues (*37*). However, comparison of **KG-3** and **KG-2** shows that substitution of the ether oxygen of **KG-2** for a methylene substantially decreases its potency in all three mutants, suggesting that the relative position of the side chain may be different for these analogues compared to GC-1. **KG-1** (EC_{50} = 48 nM) has only moderate potency and activity toward TR*β*(R316H) but is 6-fold more selective for the mutant over the wild-type receptors. Although **KG-1** lacks the potency and activity to be a practical agent for chemical rescue, these findings

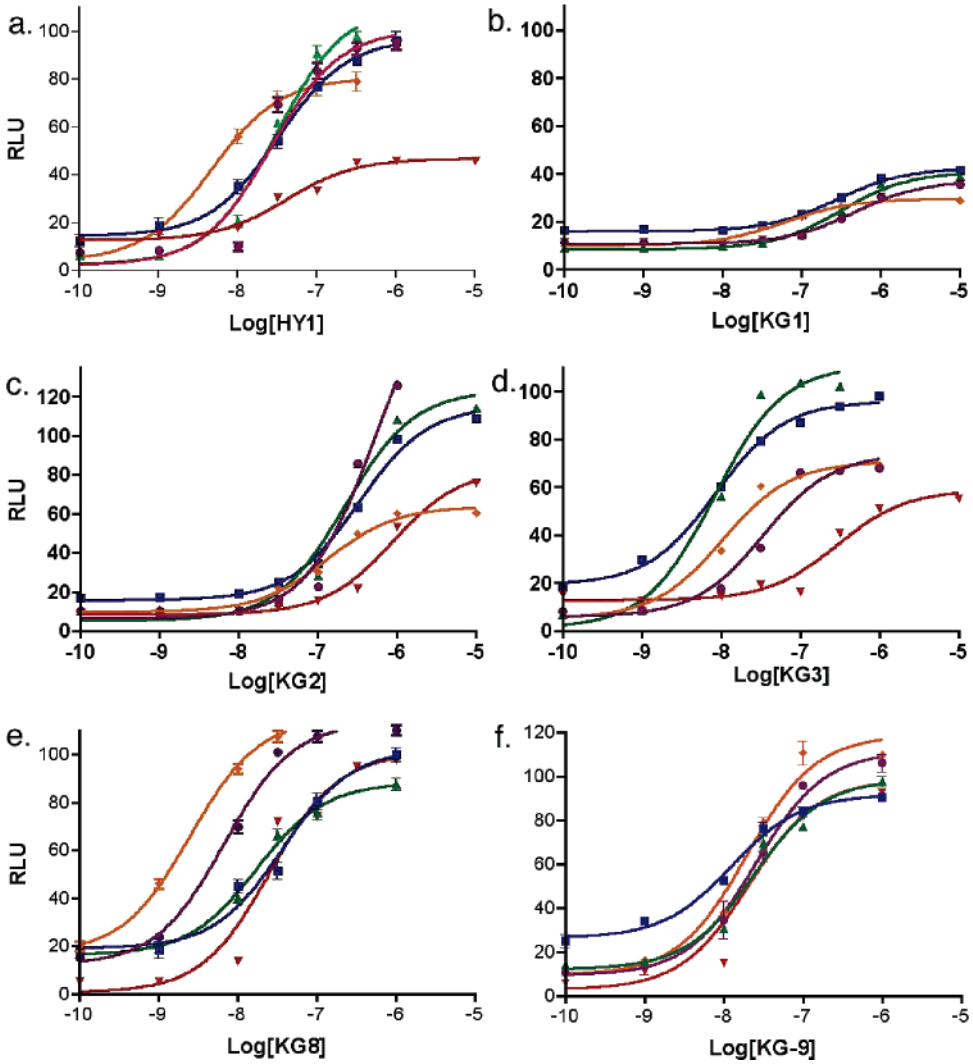


FIGURE 4: Cellular dose−response behavior of designed ligand complements with TRα(wt) (blue ■), TRβ(wt) (green ▲), and RTH-associated mutants TRβ(R320C) (gold ◆), TRβ(R320H) (magenta ●), and TRβ(R316H) (red ▼). RLU = relative light units; maximum inducible activity with TRβ(wt) and T3 was set to 100 RLU.

suggest that side-chain modification can reverse the very strong selectivity observed with T3, which is 152-fold more potent toward TRα than TRβ(R320C).

The propargyl alcohols **KG-8** and **KG-9** are potent agonists for all three mutants. Whereas **KG-9** lacks mutant/wild-type selectivity, **KG-8** shows significant selectivity, potency, and activity toward two of the three mutants. **KG-8** is more potent than T3 and HY1 with TRβ(R320C) and shows a complete reversal of receptor selectivity [being 6 times more potent in the mutant than TRβ(wt) and 12 times more potent than TRα(wt)]. Importantly, **KG-8** and **KG-9** also show good potency and recover almost full activity with R316H despite the structural perturbations noted in the crystal structure of this mutant receptor (*35*).

Of the alcohol-based ligands investigated on DR4-based reporter gene expression, HY1 and **KG-8** show significant mutant to wild-type selectivity with the R320C and/or R320H mutants. The properties of these ligands were further explored with reporter genes driven by the common consensus TREs, PAL and F2 (LAP).

Ligand Activities on TRs and Mutants R320H and R320C at PAL and F2 TREs. To determine the effects of thyromimetics on PAL-driven reporter gene expression, a synthetic PAL-3×Luc reporter gene was used in transiently transfected HEK293 cells coexpressing TRβ(R320C) or TRβ(R320H) (Table 2). The thyromimetics HY1 and **KG-8** show good potency and efficacy and significant mutant to wild-type selectivity with the PAL reporter as was observed with DR4-driven reporter gene expression (Figure 5). Interestingly, HY1 and **KG-8** are 1.5−3.5 times less potent on PAL sites than DR4 sites, which are similar to the 2.6-fold reduction in potency observed with T3 and wild-type TRβ. The mutant to wild-type selectivity is reduced somewhat for both analogues.

Toward F2-responsive reporter genes, T3 and the thyromimetics HY1 and **KG-8** had good potencies and activities similar to those observed with DR4-Luc and PAL-Luc (Figure 6). T3 and GC-1 are still more potent toward wild-type than the mutants, and **KG-8** retains mutant selectivity for both TRβ(R320C) and TRβ(R320H). HY1 has a 3-fold preference for TRβ(R320C) versus TRβ(wt); however, it does not show any selectivity when comparing TRβ(R320C) to TRα(wt) or when comparing TRβ(R320H) to TRβ(wt) (Table 3). On F2 sites, HY1 is actually 3-fold more potent toward TRα(wt) than the mutant TRβ(R320H). These findings suggest that mutant to wild-type selectivity can be

Table 2: Potencies and Efficacies of Natural and Synthetic Ligands for TRα, TRβ, TRβ(R320C), and TRβ(R320H) on PAL-Driven Reporter Gene Expression[a]

	EC_{50}, nM (% max)					EC_{50}, nM (% max)		
ligands	TRα	TRβ	R320C	α/mut	β/mut	R320H	α/mut	β/mut
T3	0.3 ± 0.1 (100)	1.2 ± 0.1 (100)	11 ± 1.0 (100)	0.03	0.11	7.0 ± 0.8 (87)	0.04	0.17
GC-1	23 ± 4.0 (72)	8.6 ± 1.0 (84)	76 ± 15 (78)	0.30	0.11	10.2 ± 1.5 (70)	2.2	0.84
HY1	93 ± 20 (60)	58 ± 10 (63)	25 ± 4.0 (85)	3.7	2.3	46 ± 10 (76)	1.6	2.0
KG-8	20 ± 3.0 (55)	29 ± 4.5 (66)	8.1 ± 1.0 (100)	2.5	3.6	11.5 ± 3.0 (82)	1.7	2.5

[a] Efficacies reported in parentheses as percentage of the maximum inducible activity observed with TRβ(wt) and T3. Mutant to wild-type selectivities, denoted as α/mut, are defined as the ratio [EC_{50} with TRα(wt)]/(EC_{50} with mutant TRβ).

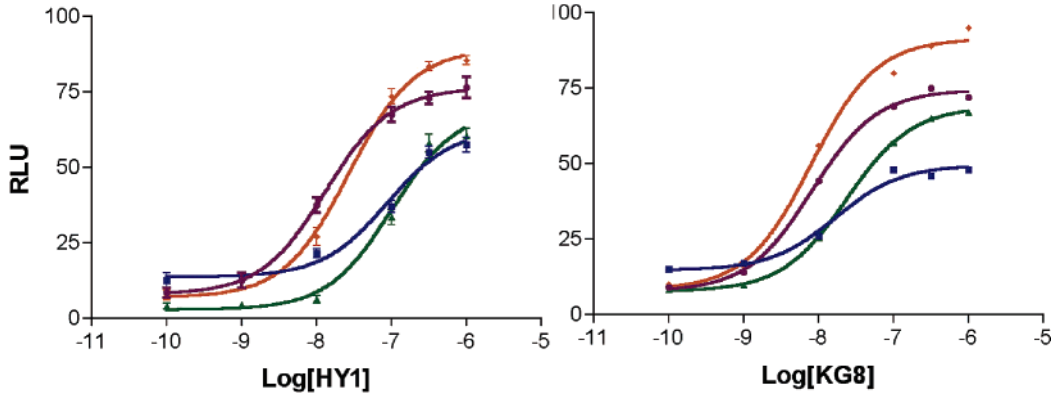


FIGURE 5: Cellular dose response of ligands on PAL response elements with TRα(wt) (blue ■), TRβ(wt) (green ▲), and RTH-associated mutants TRβ(R320C) (gold ◆) and TRβ(R320H) (magenta ●). RLU = relative light units; maximum inducible activity with TRβ(wt) and T3 was set to 100 RLU.

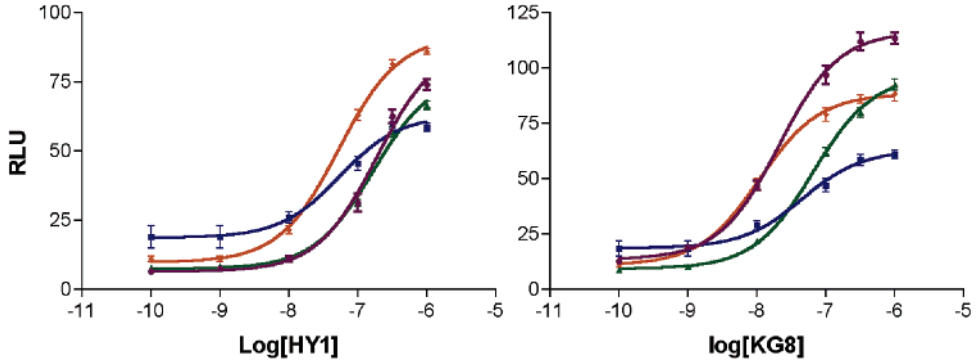


FIGURE 6: Cellular dose response of ligands on F2 response elements with TRα(wt) (blue ■), TRβ(wt) (green ▲), and RTH-associated mutants TRβ(R320C) (gold ◆) and TRβ(R320H) (magenta ●). RLU = relative light units; maximum inducible activity with TRβ(wt) and T3 was set to 100 RLU.

Table 3: Potencies and Efficacies of Natural and Synthetic Ligands for TRα, TRβ, TRβ(R320C), and TRβ(R320H) on F2-Driven Reporter Gene Expression[a]

	EC_{50}, nM (% max)					EC_{50}, nM (% max)		
ligands	TRα	TRβ	R320C	α/mut	β/mut	R320H	α/mut	β/mut
T3	0.6 ± 0.2 (92)	2.2 ± 0.2 (100)	16.7 ± 2.0 (98)	0.04	0.13	19.6 ± 2.0 (94)	0.03	0.11
GC-1	21.5 ± 3.0 (74)	10.1 ± 1.2 (94)	294 ± 40 (77)	0.07	0.03	159 ± 31 (70)	0.14	0.06
HY1	57.6 ± 10 (60)	166 ± 35 (60)	60 ± 10 (87)	0.96	2.8	158 ± 30 (72)	0.36	1.1
KG-8	47 ± 10 (65)	70 ± 12 (72)	11 ± 1.0 (85)	4.3	6.4	24 ± 5.0 (90)	2.0	2.9

[a] Potencies (EC_{50}) are reported in nanomolar. Efficacy reported as percent maximum inducible activity of wild-type TRβ with T3 is shown in parentheses. Mutant to wild-type selectivities, denoted as α/mut, are defined as the ratio [EC_{50} with TRα(wt)]/(EC_{50} with mutant TRβ).

promoter specific and highlight the importance of evaluating mutant-selective analogues on different TREs.

Effects of the Ligands on TRβ and Mutants R320H and R320C at TSH Sites. T3-dependent repression of negatively regulated genes is an important property of TRs and is principally responsible for the negative feedback regulation of thyroid hormone synthesis. Several negatively regulated genes such as those encoding the thyrotropin (TSH) and thyrotropin-releasing hormone (TRH) are crucial targets for physiological feedback by T3, through which plasma thyroid hormone levels are tightly controlled. The abnormal regulation of thyroid hormone synthesis through negative thyroid hormone response elements is believed to cause hyperthyroid conditions associated with RTH. To evaluate the effects of

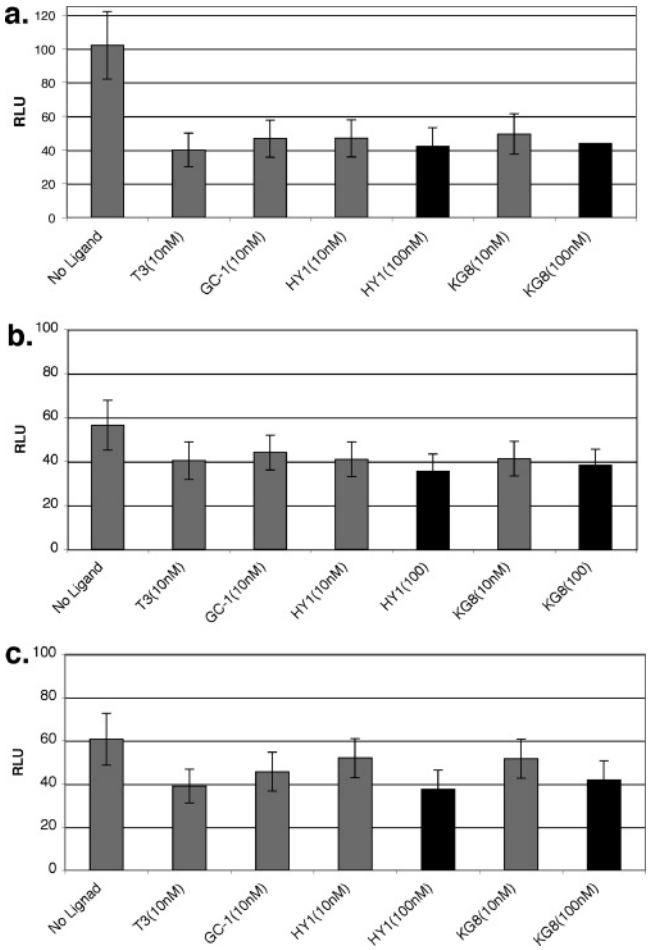


FIGURE 7: Cellular reporter gene activities with various ligands using negative response element reporter TSH-Luc cotransfected with (a) TRβ(wt), (b) TRβ(R320H), and (c) TRβ(R320C). RLU = relative light units from luciferase reporter genes normalized such that the activity observed with TRβ(wt) in the absence of ligand is equal to 100 RLU.

thyromimetics on negative response elements, a luciferase reporter gene driven by the TSH promotor, TSHα-Luc, was transiently transfected into HEK293 cells as a reporter gene.

In the presence of cotransfected TRβ(wt) receptor, TSHα-Luc activity was repressed to 40 $\pm$ 5% of basal activity upon treatment with 10 nM T3 (Figure 7). In the absence of transfected TRβ, TSHα-Luc activity was nominally stimulated (1.5-fold) by treatment with T3 (data not shown). At 10 nM, thyromimetics GC-1, HY1, and **KG-8** are able to repress TRβ(wt)-dependent TSH activity to 46−50% of basal activity (Figure 7a). The RTH mutants TRβ(R320H) and TRβ(R320C) show lower basal expression levels (56.5% and 60.7%, respectively) than TRβ(wt) in the absence of ligand. The data presented are normalized such that 100 RLU (relative light units) corresponds to TRβ(wt) in the absence of ligand. With a lower basal level of expression, the thyromimetics GC-1, HY1, and **KG-8** (10 nM) further repress TRβ(R320H)-mediated TSH reporter gene expression to 44.1%, 40.9%, and 41.2% of wild-type basal levels, respectively, compared to 39% of the wild-type basal levels with 10 nM T3. For the mutant R320C, 10 nM T3 represses TSH activity to 39.0% of wild-type basal expression, while GC-1, HY1, and **KG-8** repressed reporter gene expression less effectively to 45.6%, 51.9%, and 51.6%, respectively (Figure 7b,c). At 100 nM, the less potent analogues HY1 and **KG-8** appear to further suppress TSH-driven reporter gene expression. The smaller absolute change in ligand-induced response and larger relative error made comparisons of ligand potency by examining the full dose−response behavior of the mutants impractical.

Dominant Negative Activity on DR4 Response Elements. To assess the ability to HY1 and **KG-8** to release the dominant negative actions of TRβ mutants, equal amounts of the TRβ(wt) and of the TRβ mutant plasmid R320H or R320C (25 ng in each well) along with DR4-Luc+ reporter were cotransfected into HEK293 cells to analyze the effects of T3 and thyromimetics. Cells coexpressing both mutant and wild-type simulate the heterozygous state *in vivo*. The natural hormone T3 was 8 and 2.5 times less potent in cells coexpressing either TRβ(R320H) or TRβ(R320C) with TRβ-(wt) than in cells expressing TRβ(wt) alone, thus demonstrating the known dominant negative actions of these mutants (Table 4). Under identical conditions, GC-1 was similarly less potent in the presence of the mutant than in cells expressing only TRβ(wt). By contrast, the designed analogue HY1 is almost equipotent in cells coexpressing TRβ(R320C) as in cells expressing TRβ(wt) alone and is actually 2.5 and 3 times more potent in cells coexpressing wild-type plus TRβ(R320H) than in cells expressing only TRβR320H) or TRβ(wt). The ligand **KG-8** is similarly 6 and 3.6 times more potent in cells coexpressing the dominant mutants TRβ(R320C) and TRβ(R320H) with wild-type than with cells expressing only TRβ(wt).

Dominant Negative Actions of RTH Mutants on Negative TREs. The dominant negative actions of RTH mutants on negative hormone response elements were also evaluated. TRβ expression plasmid and the same amount of TRβ mutant plasmid R320H or R320C (25 ng in each well, 1:1 ratio) along with a TSHα-Luc reporter were cotransfected into HEK293 cells to analyze the effects of T3 and thyromimetics under conditions representing the heterozygous state found in vivo. T3 (10 nM) represses TSH activity of (TRβ + R320H) and (TRβ + R320C) to 74 $\pm$ 14% and 63 $\pm$ 12% of basal activity (Figure 8), respectively, whereas T3 can repress TSH activity of TRβ(wt) alone to 40% of its maximal expression attained in the absence of ligand (Figure 7). At 10 nM, thyromimetics HY1 and **KG-8** are able to repress TSH activity of (TRβ + R320H) to 81 $\pm$ 16% and 75 $\pm$ 15%, respectively (Figure 8a), and the TSH activity of (TRβ + R320C) to 92 $\pm$ 18% and 76 $\pm$ 16%, respectively (Figure 8b). At higher concentrations (100 nM) HY1 and **KG-8** further suppress reporter gene expression to levels similar to those observed with T3 at 10 nM. These data show that HY1 and **KG-8** can also function on negative TREs although they are not as potent as T3 or GC-1.

Receptor Binding Assay. Binding affinities of our ligands for the full-length TRs were determined by competitive radiometric binding assay using prokaryotic expression vectors pET15b-TRα, pET15b-TRβ, pET15b-TRβ (R320C), pET15b-TRβ (R320H), and pET15b-TRβ (R316H). The affinities and selectivities of ligands for TRα, TRβ, and mutants are summarized in Table 5. The binding results show that T3 and GC-1 bind the wild-type receptors much stronger than to mutants, whereas the designed thyromimetics **KG-8** and HY1 bind to R320H and R320C mutants more tightly than to the wild-type receptors, consistent with their trans-activational activities.

Table 4: Potencies of Natural and Synthetic Ligands on Dominant Negative Actions of Mutant TRβs on DR4 Responsive Reporters[a]

	EC_{50} (nM)			
	T3	GC-1	HY1	**KG-8**
TRβ(wt) + TRβ(R320C)	1.18 ± 0.83	5.98 ± 1.14	9.33 ± 1.98	5.51 ± 1.93
TRβ(wt) + TRβ(R320H)	0.37 ± 0.14	6.69 ± 1.91	11.35 ± 0.68	9.25 ± 4.35

[a] Transient transfection reporter gene activity (DR4-Luc) using equal amounts of wild-type and mutant expression plasmid.

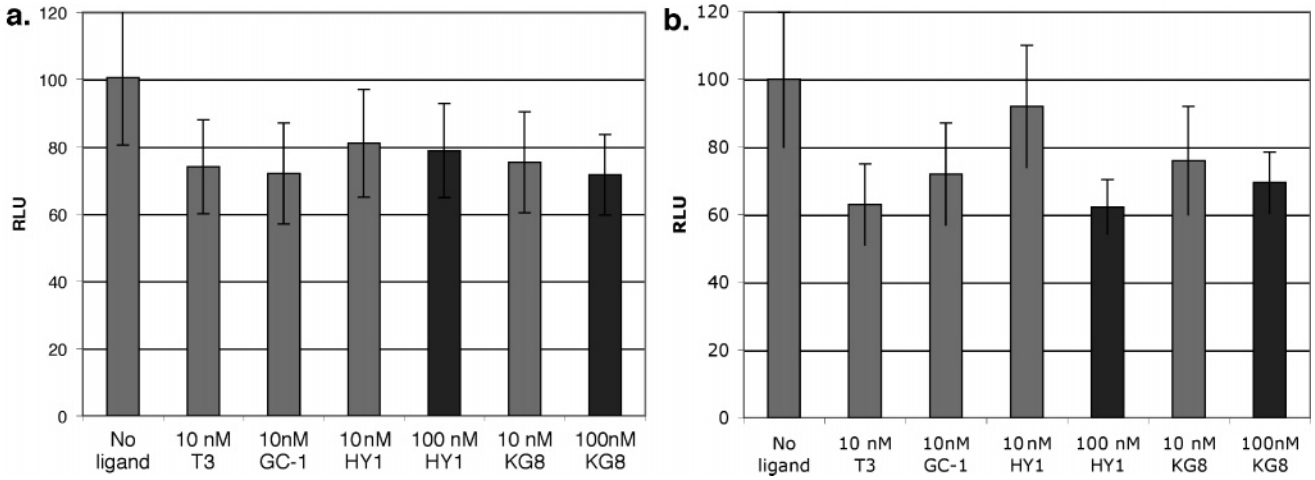


FIGURE 8: Actions of ligands (10 nM) at negative response elements (TSH-Luc) in HEK293 cells transiently cotransfected with equal amounts of TRβ(wt) and (a) TRβ(R320H) or (b) TRβ(R320C). RLU = relative light units from luciferase reporter genes independently normalized such that the activity observed in the absence of ligand is equal to 100 RLU.

Table 5: Binding Affinity for TR and Mutant Receptors Determined by Radioligand Displacement Assay

	K_d (nM)				
	TRα	TRβ	R316H	R320C	R320H
T3	0.5 ± 0.3	0.6 ± 0.3	3.3 ± 1.1	1.3 ± 0.6	1.2 ± 0.4
GC-1	16.5 ± 5.0	2.4 ± 0.8	32.5 ± 10	12.4 ± 4.0	8.7 ± 3.0
HY1	24.1 ± 5	56.6 ± 20	65.3 ± 20	10.3 ± 3.6	14.4 ± 4.0
KG-8	141 ± 50	6.6 ± 2.0	27.9 ± 8.0	8.2 ± 3.0	4.4 ± 1.1

DISCUSSION

Functional Rescue of Mutant Receptors. The design of small molecules that complement mutant proteins associated with genetic disease is a new and largely unexplored field of study. A few notable examples of molecular rescue have described molecules that assist or stabilize protein folding (*38*−*41*). In this and related studies from our laboratory, small molecules have been designed to complement mutations of nuclear hormone receptors associated with human genetic disease by rescuing function to otherwise stable proteins. In these examples, the nuclear receptor mutations alter residues that are part of the ligand−receptor interface. Though these mutations primarily affect ligand binding, they also affect the receptor's ability to structurally reorganize into a transcriptionally active conformation upon hormone binding. Therefore, both potency and activity are affected by these mutations. A priori, it is not obvious that for any given mutation a ligand can be identified which restores both potency and activity. From this and related studies it is clear that at least a subset of functionally impaired proteins associated with genetic disease can be rescued by small molecules.

Mutant-Selective Complementation. Pioneering work by DeGroot et al. has suggested that some thyroid hormone analogues may be more effective in activating RTH-associated mutants of TRβ than T3 (*24*). The TR agonists Triac (triiodothyroacetic acid) and Triprop (triiodothyropropionic acid) are more potent than T3 in some RTH-associated mutants but show even greater binding affinity toward the wild-type alleles [TRβ(wt) and TRα(wt)] than the RTH-associated mutants. Although Triac has been used clinically, its use in the treatment of RTH has been somewhat controversial (*42*, *43*).

In this study we have identified compounds that can partially, and in some cases fully, restore wild-type efficacy to three of the most common mutations associated with RTH. Though the precise molecular basis through which different receptor−ligand combinations show different efficacies has not been clearly established, the complementing ligand likely induces a conformation of the mutant receptor that more closely resembles the agonist-bound conformation of the wild type. Of particular note, the RTH-associated mutant TRβ-(R316H) was recently cocrystallized in the presence of Triac (*35*). The R316H mutant shows notable disorder to helix 1 and the receptor shows both decreased potency and activity. Despite the significant structural distortions noted in the crystal structure, the ligand **KG-8** can almost fully recover full wild-type activity, although with a lower potency than T3 has with TRβ(wt).

Many of the physiological consequences of RTH are believed to result from the imbalanced response of TRα and TRβ in peripheral and pituitary tissues. Therefore, RTH-complementing thyromimetics need not have the same potency as T3 has with TRβ(wt), so long as they are selective for the mutant over TRβ(wt) and TRα(wt). In this regard, the design strategy for developing molecular complements of the autosomal dominant mutations that cause RTH is somewhat different from that used to design hormone

analogues that complement the homozygous state associated with the recessive mutations that cause vitamin D resistant rickets (*21*, *22*). The different physiological roles of TRα and TR*β* and the critical role of TR*β* in the negative feedback regulation via negative TREs in the hypothalamus and pituitary suggest that the ideal RTH-complementing compounds should be able to selectively release the dominant negative actions of the RTH-associated TR*β* mutant without over stimulating TR*β*(wt) or TRα(wt). None of the mutant-complementing analogues in this study are as potent with the RTH-associated mutants as T3 is with TR*β*(wt). However, we have shown that HY1 and **KG-8** show a strong preference for activating the RTH-associated mutants TR*β*-(R320C) and TR*β*(R320H) over TR*β*(wt) and TRα(wt). The lower potency of the designed analogues may be due in part because they are structurally based on the GC-1 scaffold. GC-1 is significantly less potent than T3 with the targeted mutants in our cell-based assays. Significantly, several ligand−receptor pairs show high potency (EC_{50} < 30 nM), and in two examples designed ligands were more potent than T3 (and GC-1) toward the same mutant, illustrating that appropriately designed analogues can selectively improve potency of mutant receptors.

TRE Structure Influences Potency, Efficacy, and Selectivity. Two different mechanisms have been proposed to account for the dominant negative actions of the mutant of TR*β*s depending on their roles in either homodimers or heterodimers with RXR. As heterodimers, mutant TR*β*/RXR heterodimers can compete with TR*β*(wt)/RXR heterodimers. As homodimers, mutant TRs can form mutant/wild-type or mutant/mutant dimers of reduced activity. TR*β*/RXR heterodimers bind more strongly to DR4 and Pal TREs than TR*β*/TR*β* homodimers; however, the F2 TRE strongly favors the TR*β* homodimers, suggesting that both mechanisms are potentially operative in RTH. Although some studies have shown that the addition of mutations that selectively reduce a receptor's ability to heterodimerize reduces the dominant negative actions of RTH mutants, some RTH mutants that preferentially form heterodimers over homodimers also show reduced dominant negative activity (*12*, *44*−*46*). At this point it is not clear if any one mechanism of dominant negative action contributes more significantly to the phenotype of RTH. In this study we find that complementing analogues HY1 and **KG-8** can selectively rescue activity to RTH mutants on DR4, PAL, and F2 promotors, demonstrating that this approach to molecular rescue can function on promoters that favor either heterodimers or homodimers. The magnitude of mutant to wild-type selectivity, however, is not the same on the different promoters studied. Of note, HY1, which slightly favors the mutant TR*β*(R320H) over TRα(wt) on both DR4 and PAL promoters, has the opposite selectivity on the F2 promotor, favoring TRα over TR*β*(R320H) by almost 3-fold. The observed differences in potency on different TREs are not limited to RTH mutants or synthetic ligands as even T3 with TR*β*(wt) is almost 5-fold less potent on F2 as it is on DR4.

Additionally, the most promising analogues HY1 and **KG-8** were evaluated on the negative TRE of TSHα. Interestingly, the mutants TR*β*(R320C) and TR*β*(R320H) have lower basal transcription levels that can be lowered further by the addition of T3 or synthetic ligand. Not surprisingly, the synthetic analogues, which are more selective but less potent than T3 on positive TREs, require higher concentrations to elicit an equivalent response.

It is important to recognize that receptor dimerization, DNA binding, and coactivator recruitment occur as a cooperative process. Indeed, in solution, TR*β* dimers have been shown to be affected by T3 binding, and the hormone responsiveness of some steroid receptors can be significantly modulated by altering coactivator and/or corepressor concentrations (*47*). It has been proposed for other NHRs that receptors may adopt different conformations on different promotors and consequently interact with different efficiencies toward different coactivators (*48*, *49*). Taken together, these findings suggest that the potency and efficacy of different ligand−receptor pairs need not be the same on all TREs. Therefore, molecular complements of RTH may need to be evaluated on all representative promotor contexts.

Defining Rules for Molecular Complementation. The three mutations examined in this study represent three of the most common mutations found in RTH patients. Interestingly, all three mutations are chemically similar in that they affect the cluster of basic residues used to bind the carboxylate of T3, by converting a highly basic arginine to a neutral, polar residue. Initially, we demonstrated that the neutral alcohol HY1 was a potent, mutant-selective agonist for the RTH-associated mutation TR*β*(R320C). Here we show that HY1 and related alcohol side-chain thyromimetics can also serve as mutant-selective complements to related Arg → neutral mutations in the carboxylate-binding pocket of T3. The use of neutral polar ligands to selectively complement these charge-neutralizing mutations of the basic carboxylate-binding arginine cluster appears to be somewhat general. Therefore, although it is unlikely that any single compound might work effectively in every mutation affecting the carboxylate-binding arginine cluster, compounds such as **KG-8** are potent and show substantial improvements in mutant versus wild-type selectivity for all three mutants examined in this study. Through a better understanding of the molecular basis for mutant versus wild-type receptor selectivity, we envision that the same general ligand design strategy may be used in similar mutations affecting the basic polar cluster in TR as well as other nuclear receptors associated with human disease. Although the clinical applications of potential mutant-specific analogues remain many years away, this study demonstrates in vitro that custom-designed analogues have the ability to selectively rescue the dominant negative actions of RTH mutations in a manner consistent with our understanding of the molecular basis of the disease.

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REFERENCES

1. Hulbert, A. J. (2000) Thyroid hormones and their effects: A new perspective, *Biol. Rev. 75*, 519−631.
2. Yen, P. M. (2001) Physiological and molecular basis of thyroid hormone action, *Physiol. Rev. 81*, 1097−1142.
3. Greenspan, F. S. (1994) in *Basic and Clinical Endocrinology* (Greenspan, F. S., and Baxter, J. D., Eds.) pp 160−226, Appleton & Lange, Norwalk, VA.

4. Apriletti, J. W., Ribeiro, R. C., Wagner, R. L., Feng, W., Webb, P., Kushner, P. J., West, B. L., Nilsson, S., Scanlan, T. S., Fletterick, R. J., and Baxter, J. D. (1998) Molecular and structural biology of thyroid hormone receptors, *Clin. Exp. Pharmacol. Physiol., Suppl. 25*, S2−S11.
5. Klein, I., and Ojamaa, K. (2001) Mechanisms of disease: Thyroid hormone and the cardiovascular system, *N. Engl. J. Med. 344*, 501−509.
6. Refetoff, S., Weiss, R. E., and Usala, S. J. (1993) The syndromes of resistance to thyroid hormone, *Endocr. Rev. 14*, 348−399.
7. Baxter, J. D., Dillmann, W. H., West, B. L., Huber, R., Furlow, J. D., Fletterick, R. J., Webb, P., Apriletti, J. W., and Scanlan, T. S. (2001) Selective modulation of thyroid hormone receptor action, *J. Steroid Biochem. Mol. Biol. 76*, 31−42.
8. Kahaly, G. J., Matthews, C. H., Mohr-Kahaly, S., Richards, C. A., and Chatterjee, V. K. K. (2002) Cardiac involvement in thyroid hormone resistance, *J. Clin. Endocrinol. Metab. 87*, 204−212.
9. Baxter, J. D., Webb, P., Grover, G., and Scanlan, T. S. (2004) Selective activation of thyroid hormone signaling pathways by GC-1: A new approach to controlling cholesterol and body weight, *Trends Endocrinol. Metab. 15*, 154−157.
10. Dupre, S. M., Guissouma, H., Flamant, F., Seugnet, I., Scanlan, T. S., Baxter, J. D., Samarut, J., Demeneix, B. A., and Becker, N. (2004) Both thyroid hormone receptor TR(beta) 1 and TR(beta) 2 isoforms contribute to the regulation of hypothalamic thyrotropin-releasing hormone, *Endocrinology 145*, 2337−2345.
11. Yen, P. M. (2003) Molecular basis of resistance to thyroid hormone, *Trends Endocrinol. Metab. 14*, 327−333.
12. Collingwood, T. N., Adams, M., Tone, Y., and Chatterjee, V. K. K. (1994) Spectrum of transcriptional, dimerization, and dominant-negative properties of 20 different mutant thyroid-hormone beta-receptors in thyroid-hormone resistance syndrome, *Mol. Endocrinol. 8*, 1262−1277.
13. Chatterjee, V. K., Nagaya, T., Madison, L. D., Datta, S., Rentoumis, A., and Jameson, J. L. (1991) Thyroid hormone resistance syndrome. Inhibition of normal receptor function by mutant thyroid hormone receptors, *J. Clin. Invest. 87*, 1977−1984.
14. Liu, Y., Takeshita, A., Misiti, S., Chin, W. W., and Yen, P. M. (1998) Lack of coactivator interaction can be a mechanism for dominant negative activity by mutant thyroid hormone receptors, *Endocrinology 139*, 4197−4204.
15. Piedrafita, F. J., Ortiz, M. A., and Pfahl, M. (1995) Thyroid hormone receptor-beta mutants associated with generalized resistance to thyroid hormone show defects in their ligand-sensitive repression function, *Mol. Endocrinol. 9*, 1533−1548.
16. Takeda, K., Balzano, S., Sakurai, A., DeGroot, L. J., and Refetoff, S. (1991) Screening of nineteen unrelated families with generalized resistance to thyroid hormone for known point mutations in the thyroid hormone receptor beta gene and the detection of a new mutation, *J. Clin. Invest. 87*, 496−502.
17. Weiss, R. E., Hayashi, Y., Nagaya, T., Petty, K. J., Murata, Y., Tunca, H., Seo, H., and Refetoff, S. (1996) Dominant inheritance of resistance to thyroid hormone not linked to defects in the thyroid hormone receptor alpha or beta genes may be due to a defective cofactor, *J. Clin. Endocrinol. Metab. 81*, 4196−4203.
18. Yen, P. M., and Cheng, S. Y. (2003) Germline and somatic thyroid hormone receptor mutations in man, *J. Endocrinol. Invest. 26*, 780−787.
19. Cheng, S. Y. (2003) Thyroid hormone mutations in cancer, *Mol. Cell. Endocrinol. 213*, 23−30.
20. Ye, H. F., O'Reilly, K. E., and Koh, J. T. (2001) A subtype-selective thyromimetic designed to bind a mutant thyroid hormone receptor implicated in resistance to thyroid hormone, *J. Am. Chem. Soc. 123*, 1521−1522.
21. Swann, S. L., Bergh, J., Farach-Carson, M. C., Ocasio, C. A., and Koh, J. T. (2002) Structure based design of selective agonists for a rickets-associated mutant of the vitamin d receptor, *J. Am. Chem. Soc. 124*, 13795−13805.
22. Swann, S. L., Bergh, J. J., Farach-Carson, M. C., and Koh, J. T. (2002) Rational design of vitamin D3 analogs which selectively restore activity to a vitamin D receptor mutant associated with rickets, *Org. Lett. 4*, 3863−3866.
23. Gardezi, S. A., Nguyen, C., Malloy, P. J., Posner, G. H., Feldman, D., and Peleg, S. (2001) A rationale for treatment of hereditary vitamin D-resistant rickets with analogs of 1 alpha,25-dihydroxy-vitamin D-3, *J. Biol. Chem. 276*, 29148−29156.
24. Takeda, T., Suzuki, S., Liu, R. T., and DeGroot, L. J. (1995) Triiodothyroacetic acid has unique potential for therapy of resistance to thyroid hormone, *J. Clin. Endocrinol. Metab. 80*, 2033−2040.
25. Hayashi, Y., Sunthornthepvarakul, T., and Refetoff, S. (1994) Mutations of CpG dinucleotides located in the triiodothyronine (t-3)-binding domain of the thyroid-hormone receptor TR(beta)-gene that appears to be devoid of natural mutations may not be detected because they are unlikely to produce the clinical phenotype of resistance to thyroid-hormone, *J. Clin. Invest. 94*, 607−615.
26. McMartin, C., and Bohacek, R. S. (1997) Qxp: Powerful, rapid computer algorithyms for structure-based drug design, *J. Comput.-Aided Mol. Des. 11*, 333−344.
27. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Structure and specificity of nuclear receptor-coactivator interactions, *Genes Dev. 12*, 3343−3356.
28. Wagner, R. L., Huber, B. R., Shiau, A. K., Kelly, A., Lima, S. T. C., Scanlan, T. S., Apriletti, J. W., Baxter, J. D., West, B. L., and Fletterick, R. J. (2001) Hormone selectivity in thyroid hormone receptors, *Mol. Endocrinol. 15*, 398−410.
29. Wagner, R., Apriletti, J. W., McGarth, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995) A structural role for hormone in the thyroid hormone receptor, *Nature 378*, 690−697.
30. Chiellini, G., Apriletti, J. W., al Yoshihara, H., Baxter, J. D., Ribeiro, R. C., and Scanlan, T. S. (1998) A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor, *Chem. Biol. 5*, 299−306.
31. Scafonas, A., Wolfgang, C. L., Gabriel, J. L., Soprano, K. J., and Soprano, D. R. (1997) Differential role of homologous positively charged amino acid residues for ligand binding in retinoic acid receptor alpha compared with retinoic acid receptor beta, *J. Biol. Chem. 272*, 11244−11249.
32. Weatherman, R. V., Fletterick, R. J., and Scanlan, T. S. (1999) Nuclear-receptor ligands and ligand-binding domains, *Annu. Rev. Biochem. 68*, 559−581.
33. Burris, T. P., and McCabe, E. R. B. (2001) *Nuclear Receptors and Genetic Disease*, Academic Press, San Diego.
34. Huber, B. R., Desclozeaux, M., West, B. L., Cunha-Lima, S. T., Nguyen, H. T., Baxter, J. D., Ingraham, H. A., and Fletterick, R. J. (2003) Thyroid hormone receptor-beta mutations conferring hormone resistance and reduced corepressor release exhibit decreased stability in the n-terminal ligand-binding domain, *Mol. Endocrinol. 17*, 107−116.
35. Huber, B. R., Sandler, B., West, B. L., Lima, S. T. C., Nguyen, H. T., Apriletti, J. W., Baxter, J. D., and Fletterick, R. J. (2003) Two resistance to thyroid hormone mutants with impaired hormone binding, *Mol. Endocrinol. 17*, 643−652.
36. Chiellini, G., Nguyen, N. H., Apriletti, J. W., Baxter, J. D., and Scanlan, T. S. (2002) Synthesis and biological activity of novel thyroid hormone analogues: 5′-aryl substituted GC-1 derivatives, *Bioorg. Med. Chem. 10*, 333−346.
37. Yoshihara, H. A. I., Apriletti, J. W., Baxter, J. D., and Scanlan, T. S. (2003) Structural determinants of selective thyromimetics, *J. Med. Chem. 46*, 3152−3161.
38. Hammarstrom, P., Wiseman, R. L., Powers, E. T., and Kelly, J. W. (2003) Prevention of transtyretin amyloid disease by changing protein misfolding energetics, *Science 299*, 713−716.
39. Foster, B. A., Coffey, H. A., Morin, M. J., and Rastinejad, F. (1999) Pharmacological rescue of mutant p53 conformation and function, *Science 286*, 2507−2510.
40. Noorwez, S. M., Kuksa, V., Imanishi, Y., Shu, L., Filipek, S., Palczewski, K., and Kauushal, S. (2003) Pharmacological chaperone-mediated in vivo folding and stabilization of the p23h-opsin mutant associated with autosomal dominant retinitis pigmentosa, *J. Biol. Chem. 278*, 14442−14450.
41. Bernier, V., Lacace, M., Bichet, D. G., and Bouvier, M. (2004) Pharmacological chaperones: Potential treatment for conformational diseases, *Trends Endocrinol. Metab. 15*, 222−228.
42. Radetti, G., Persani, L., Molinaro, G., Mannavola, D., Cortelazzi, D., Chatterjee, V. K. K., and BeckPeccoz, P. (1997) Clinical and hormonal outcome after two years of triiodothyroacetic acid treatment in a child with thyroid hormone resistance, *Thyroid 7*, 775−778.
43. Asteria, C., Rajanayagam, O., Collingwood, T. N., Persani, L., Romoli, R., Mannavola, D., Zamperini, P., Buzi, F., Ciralli, F.,

Chatterjee, V. K. K., and Beck-Peccoz, P. (1999) Prenatal diagnosis of thyroid hormone resistance, *J. Clin. Endocrinol. Metab. 84*, 405−410.
44. Nagaya, T., Eberhardt, N. L., and Jameson, J. L. (1993) Thyroid-hormone resistance syndrome—correlation of dominant-negative activity and location of mutations, *J. Clin. Endocrinol. Metab. 77*, 982−990.
45. Nagaya, T., and Jameson, J. L. (1993) Thyroid-hormone receptor dimerization is required for dominant-negative inhibition by mutations that cause thyroid-hormone resistance, *J. Biol. Chem. 268*, 15766−15771.
46. Hao, E. H., Menke, J. B., Smith, A. M., Jones, C., Geffner, M. E., Hershman, J. M., Wuerth, J. P., Samuels, H. H., Ways, D. K., and Usala, S. J. (1994) Divergent dimerization properties of mutant beta-1 thyroid-hormone receptors are associated with different dominant-negative activities, *Mol. Endocrinol. 8*, 841−851.
47. Szapary, D., Huang, Y., and Simons, S. S., Jr. (1999) Opposing effects of corepressor and coactivators in determining the dose−response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression, *Mol. Endocrinol. 13*, 2108−2121.
48. Loven, M. A., Likhite, V. S., Choi, I., and Nardulli, A. M. (2001) Estrogen response elements alter coactivator recruitment through allosteric modulation of estrogen receptor beta conformation, *J. Biol. Chem. 276*, 45282−45288.
49. Wood, J. R., Likhite, V. S., Loven, M. A., and Nardulli, A. M. (2001) Allosteric modulation of estrogen receptor conformation by different estrogen response elements, *Mol. Endocrinol. 15*, 1114−1126.

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