A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor

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Background: Thyroid hormones regulate many different physiological processes in different tissues in vertebrates. Most of the actions of thyroid hormones are mediated by the thyroid hormone receptor (TR), which is a member of the nuclear receptor superfamily of ligand-activated transcription regulators. There are two different genes that encode two different TRs, TRα and TRβ, and these two TRs are often co-expressed at different levels in different tissues. Most thyroid hormones do not discriminate between the two TRs and bind both with similar affinities.

Results: We have designed and synthesized a thyroid hormone analog that has high affinity for the TRs and is selective in both binding and activation functions for TRβ over TRα. The compound, GC-1, was initially designed to solve synthetic problems that limit thyroid hormone analog preparation, and contains several structural changes with respect to the natural hormone 3,5,3′-triiodo-L-thyronine (T_3). These changes include replacement of the three iodines with methyl and isopropyl groups, replacement of the biaryl ether linkage with a methylene linkage, and replacement of the amino-acid sidechain with an oxyacetic-acid sidechain.

Conclusions: The results of this study show that GC-1 is a member of a new class of thyromimetic compounds that are more synthetically accessible than traditional thyromimetics and have potentially useful receptor binding and activation properties. The TRβ selectivity of GC-1 is particularly interesting and suggests that GC-1 might be a useful *in vivo* probe for studying the physiological roles of the different thyroid hormone receptor isoforms.

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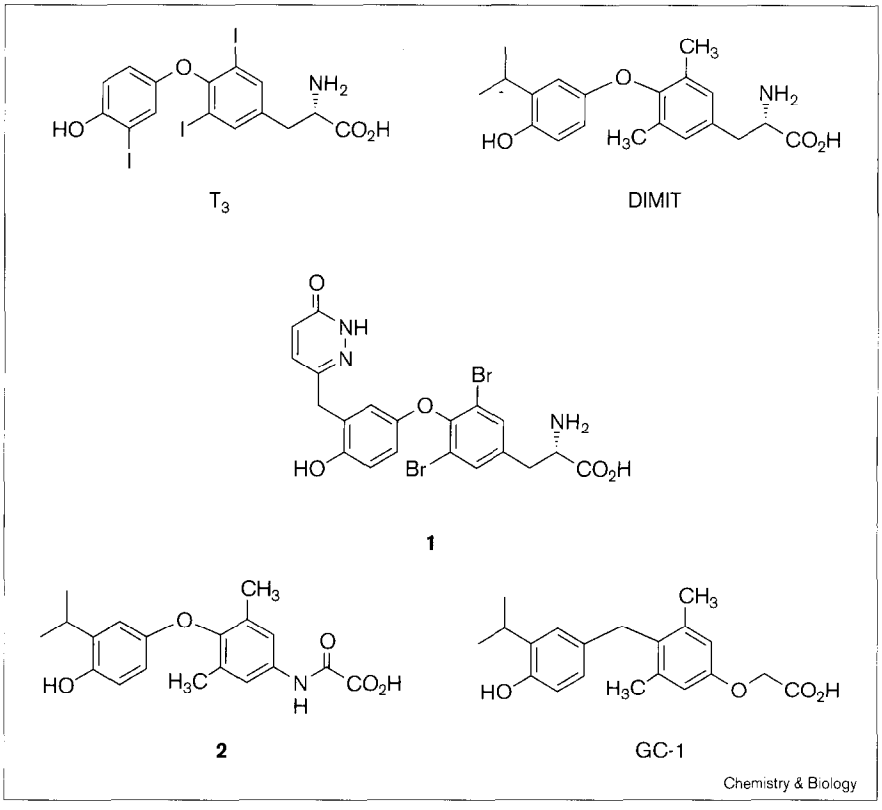
Introduction

Thyroid hormones, of which 3,5,3′-triiodo-L-thyronine (T_3, Figure 1) is the major active form, are important endocrine signaling molecules in vertebrates that exert a multitude of physiological effects [1]. These influences affect fetal development, lipid and carbohydrate metabolism, and cardiovascular, pulmonary and neuromuscular status. Most of the physiological actions of T_3 result from influences on transcription of T_3-responsive genes that are mediated through thyroid hormone receptors (TRs). The TR is a member of a superfamily of nuclear ligand-regulated transcription factors that includes receptors for steroid hormones such as estrogens and glucocorticoids, as well as those, like the TR, that are regulated by nonsteroidal ligands such as the retinoic acid and vitamin D receptors [2–5]. Nuclear receptor family members share a common domain organization that includes a central highly conserved DNA-binding domain (DBD), a less conserved carboxy-terminal ligand-binding domain (LBD), and an amino-terminal domain that varies greatly in terms of size and amino acid composition. Genes regulated by T_3 contain a regulatory DNA sequence linked to their promoter called a thyroid response element (TRE). The TR is localized in the nucleus and binds to the TRE, commonly as a heterodimer with the retinoid X receptor (RXR) [3]. In the absence of T_3, the TR–RXR heterodimer can repress gene transcription [6]. Activation of transcription occurs when T_3 permeates the nucleus and binds to the TR. Recent structural data on the liganded TR LBD reveals a unique internal-binding mode for the hormone ligand T_3; the hormone is completely buried in the solvent occluded hydrophobic core of the LBD [7]. Thus, the hormone plays a structural role in transcriptional activation by correctly shaping an active conformation of the TR. The internal ligand-binding mode has also been observed for other nuclear receptors including the retinoic acid receptor (RAR) [8] and the estrogen receptor (ER) [9] and is most likely general for the entire superfamily.

In vertebrates there are two different TR subtype genes, TRα and TRβ [10], which reside on chromosomes 17 and 3, respectively. The polypeptide sequences of TRα and TRβ are 82% identical in the LBD and 86% identical in the DBD. TR diversity is enhanced by the presence of additional isoforms from the α and β genes. Alternative

Figure 1



Agonist ligands for the thyroid hormone receptor. Thyroid hormone is 3,5,3′-triiodo-L-thyronine (T_3). DIMIT (dimethyl-isopropyl-T_3), **1**, **2**, and GC-1 are synthetic thyromimetic compounds.

splicing of the most 3′ exon of the $TR\alpha_1$ gene gives rise to a non-T_3-binding isoform, $TR\alpha_2$, that lacks the carboxy-terminal 40 amino acids of human $TR\alpha_1$ but contains an additional 120 amino acids compared to the human $TR\alpha_1$. Alternative usage of a 5′ exon in the $TR\beta_1$ gene gives rise to $TR\beta_2$, which is identical to $TR\beta_1$ except that it contains a unique amino-terminal domain. Both TR subtypes and the resulting isoforms are widely distributed, although there are differences in concentrations in various tissues [11]. For example, $TR\alpha_1$ and $TR\alpha_2$ are most abundant in skeletal muscle and in brain, respectively, whereas the $TR\beta_1$ is more homogeneously distributed but found in high levels in liver, kidney, and brain. In contrast, the $TR\beta_2$ is mostly restricted to the pituitary although its mRNA expression has been reported in other regions such as the hypothalamus and hippocampus [11].

Several of the effects of thyroid hormones in excess might be beneficial in medical therapy. These include a lowering of the serum cholesterol levels, weight loss, and improvement in myocardial contractility [1]. These beneficial influences are counterbalanced by deleterious effects, however, especially tachycardia and a tendency to develop atrial arrhythmias. It follows that a thyroid hormone analog that retained the beneficial influences of T_3, but was devoid of the deleterious influences, such as on the heart rate, would be a useful agent for medical therapy.

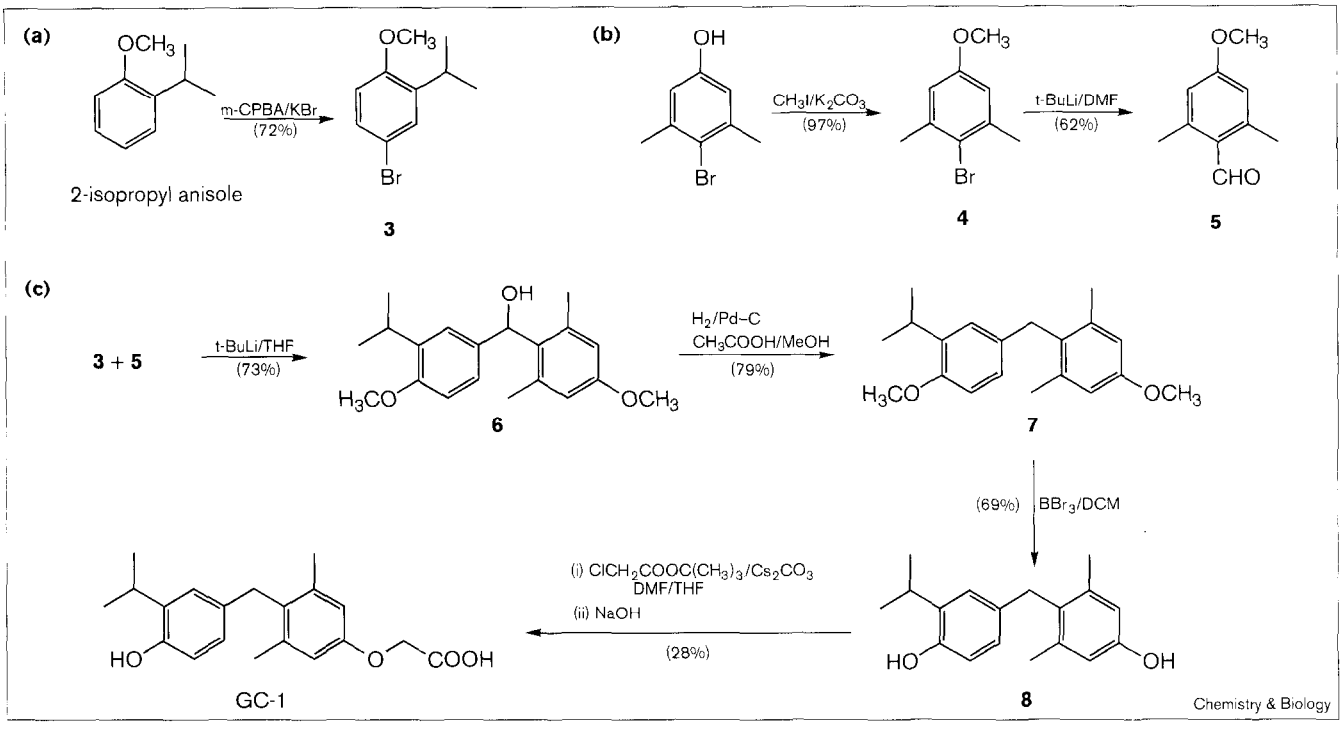
Two thyromimetic lead compounds (**1** and **2**, Figure 1) that can separate the liver and heart effects have been reported [12–16]. Interestingly, **2** shows selectivity for activation of $TR\beta_1$ compared to $TR\alpha_1$ [16]. Such selectivity suggests an approach involving the development of receptor-subtype-specific ligands because receptor subtype selectivity could correlate with tissue-specific *in vivo* thyromimetic effects. In this paper we describe the synthesis and novel properties of a TR analog GC-1 (Figure 1), which is a halogen-free high affinity ligand that is selective for TRβ over TRα.

Results

Ligand design

The initial goal of this research was to design a thyroid hormone analog that would be amenable to extensive analog production. There is a relatively large body of structure–activity data on thyroid hormone analogs that highlights required structural features for high-affinity TR ligands [17,18]. There are structural features of the natural hormone T_3 that present significant challenges to chemical synthesis and thus limit analog design, however. Although hydrophobic substituents at the 3,5 and 3′ positions are required for high-affinity binding to the TR, the iodine substituents of T_3 are susceptible to reductive deiodination, which places restrictions on the types of reagents that can be used after the iodines atoms are

Figure 2



Synthetic route used for the preparation of GC-1. **(a)** Synthesis of **3**. **(b)** Synthesis of **5**. **(c)** Synthesis of GC-1 from **3** and **5**. Isolated yields are indicated in parentheses.

installed. In addition, formation of biaryl ethers remains a significant problem in organic synthesis and the biaryl-ether linkage in T_3 is a particularly difficult one to form because of the steric interactions that result from the 3,5 substituents on the aromatic ring bearing the alanine sidechain. Motivated by these synthetic considerations, we designed the compound GC-1 (Figure 1) which is a shape mimic of T_3 that contains all the essential molecular recognition components to insure reasonable affinity for the TR. The structural differences between GC-1 and T_3 are: firstly, replacement of the 3,5,3′-iodo groups with chemically inert methyl and isopropyl substituents; secondly, replacement of the biaryl-ether linkage with a methylene linkage; and thirdly, replacement of the amino-acid substituent at the 1 position with an oxyacetic acid substituent. Thus, GC-1 satisfies all the essential design criteria of a thyromimetic agent and can be synthesized using a convergent route that is amenable to analog production.

Ligand synthesis

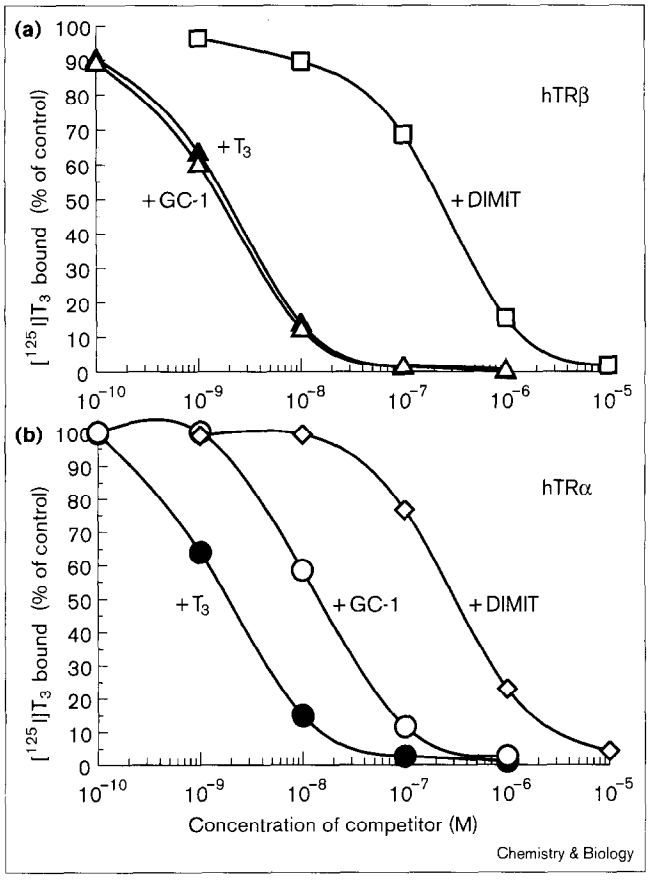
The chemical synthesis of GC-1 is outlined in Figure 2. The two aryl halves of GC-1 are constructed first: 2-isopropyl anisole [19] is brominated giving bromide **3**, and 4-bromo-3,5-dimethyl phenol is methylated and formylated to provide the aldehyde **5**. Bromide **3** and aldehyde **5** are then coupled via the addition of lithiated **3** to aldehyde **5** to give the biaryl alcohol **6**. This C–C bond-forming coupling reaction proceeds in very good overall yield and we suspect that this process will be far more tolerant to different substituents than the analogous biaryl-ether-forming reactions. Hydrogenolysis of alcohol **6** provides the bis-anisole **7**, which can then be demethylated to the bis-phenol **8** using boron tribromide.

The final step of the synthesis involves mono-alkylation of **8** with an α-haloacetate derivative and we expected that this alkylation would proceed selectively to favor the desired 1-alkylated product over the 4′-alkylated product. The 4′-hydroxyl is more sterically hindered than the 1-hydroxyl because of the neighboring isopropyl substituent, and alkylations on similar bis-phenol compounds were reported to proceed with such selectivity [15]. Unfortunately we were unable to find conditions that led to selective alkylation and this reaction always led to a mixture of both 1- and 4′-alkylated product, as well as the bis-alkylated product. The best conditions for the alkylation reaction involved the use of the sterically hindered α-chloro *t*-butylacetate providing GC-1 in 28% isolated yield after basic hydrolysis of the *t*-butyl ester.

Ligand binding to TRα and TRβ

The binding affinity of GC-1 to TRs was measured using a radioligand-displacement assay. Purified human $TR\alpha_1$

Figure 3



Competition radioligand displacement assay of ligand affinity for human TRβ$_1$ **(a)** and human TRα$_1$ **(b)**. A fixed concentration of [^{125}I]T_3 was used as the radioligand with the indicated range of concentrations of nonradioactive competitor ligand (x-axis). The competition data were fit to the equations of Swillens [22] using the GraphPad Prism computer program.

and TRβ$_1$ were analyzed independently using a fixed concentration of [^{125}I]T_3 in the presence of a range of concentrations of GC-1. GC-1 bound to TRβ$_1$ with approximately the same affinity as T_3 (Figure 3a), whereas GC-1 bound to TRα$_1$ with approximately 0.1 times the affinity of T_3 (Figure 3b). T_3 binds to both TRα$_1$ and TRβ$_1$ with similar affinities, with an apparent equilibrium dissociation constant (K_D) of 58 pM for hTRα and 81 pM for hTRβ (Table 1). For GC-1, the K_D is 67 pM for TRβ$_1$ and 440 pM for TRα$_1$ (Table 1).

As a comparison, the iodine-free ligand 3,5-dimethyl-3′-isopropyl-L-thyronine (DIMIT) was also tested for binding to TRα$_1$ and TRβ$_1$ using this assay. DIMIT was found to bind both TRs with an affinity approximately 0.01 times that of T_3 (Figures 3a,b). In addition to the weaker affinity, DIMIT showed no difference in affinity between TRα$_1$ and TRβ$_1$; the K_D values for DIMIT–TRα$_1$ and DIMIT–TRβ$_1$ were both 10 nM (Table 1).

Table 1

Binding affinity of hTRα$_1$ and hTRβ$_1$ for T_3, GC-1 and DIMIT.

Ligand	K_D ± SE (pM) hTRα$_1$	hTRβ$_1$	K_D(TRα$_1$)/K_D(TRβ$_1$)
T_3	58 ± 12	81 ± 12	0.7
GC-1	440 ± 120	67 ± 4	6.6
DIMIT	9700 ± 1500	9800 ± 1700	1.0

The K_D and standard error (SE) values were calculated by fitting the competition data in Figure 3 to the equations of Swillens [22] using the GraphPad prism computer program.

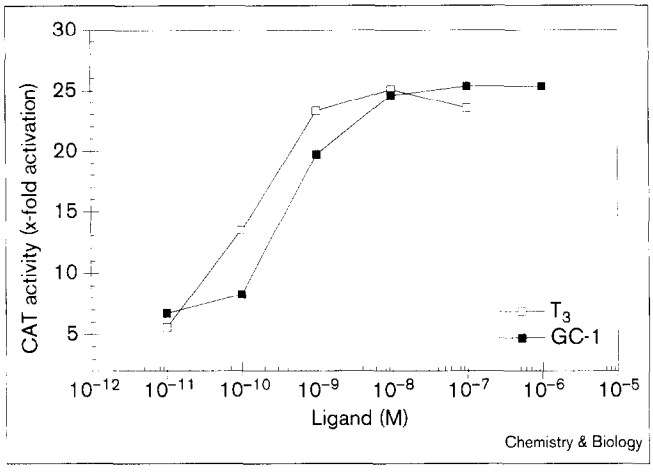
Ligand activation of transcription

The ligand activation properties of GC-1 were analyzed in mammalian cells through the use of a reporter-gene assay. GC-1 was first tested in cultured rat pituitary GC cells that endogenously express TR, with TRβ$_2$ being the predominant isoform [20]. These cells were transfected with a reporter plasmid that contained two copies of a TRE, each with two TR-binding half sites oriented as direct repeats separated by four nucleotides (DR4) driving transcription of the chloramphenicol acetyl transferase (CAT) gene, and were treated with various concentrations of GC-1 or T_3. On the basis of CAT activity, GC-1 was found to be a full TR agonist with a dose-dependent potency similar to that of T_3 (Figure 4). We next asked whether the TRβ$_1$ selectivity apparent in the *in vitro* binding experiments would also be seen in a cellular transactivation experiment. For this experiment, we used HeLa cells, which do not express significant levels of endogenous TRs. HeLa cells were transfected with either TRα$_1$ or TRβ$_1$ expression plasmids along with a TRE–luciferase reporter plasmid and treated with various concentrations of GC-1 or T_3. Six different activation experiments were performed for TRα$_1$ and TRβ$_1$ and the mean values of the data ± standard deviation were analyzed. The results indicate that, with TRβ$_1$, GC-1 is a full agonist with an EC_{50} (concentration of ligand required for one-half maximum activation) that is approximately five times greater than that of T_3 (Figure 5a), whereas with TRα$_1$, GC-1 is a full agonist with an EC_{50} that is approximately 100 times greater than that of T_3 (Figure 5b). Thus, GC-1 is a selective ligand for TRβ$_1$ over TRα$_1$ in both receptor-binding and ligand-activation functions.

Discussion

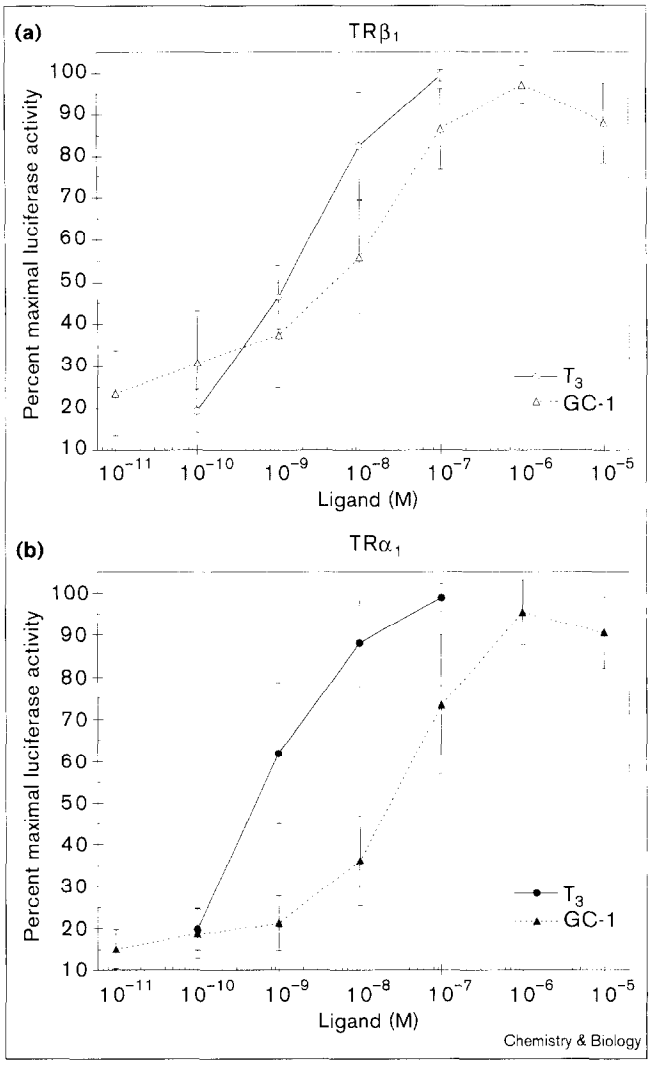
The studies reported here reflect our initial efforts to develop ligands that have TR subtype selectivity in their binding and actions. There are, however, inherent synthetic difficulties in developing structurally diverse collections of thyromimetic analogs based on the structure of T_3. The three iodine atoms of T_3 are sensitive to deiodination, and, as a result of steric bulk and electron withdrawal, they limit substitution at certain positions in the aromatic rings. In addition, the biaryl-ether linkage between the aromatic rings poses difficulties because biaryl-ether-forming reactions are not generally robust

Figure 4



Ligand activation of a TRE-driven reporter gene in GC cells. The chloramphenicol acetyltransferase gene was used as the reporter and T_3 and GC-1 were used as ligands. GC cells express endogenous TRs with $TR\beta_2$ as the predominant isoform.

Figure 5



TR subtype selectivity measured by ligand activation of a TRE-driven reporter gene in HeLa cells. HeLa cells were transfected with expression plasmids for either $TR\beta_1$ **(a)** or $TR\alpha_1$ **(b)** and a reporter plasmid containing a TRE-driven luciferase gene. The doubly transfected cells were treated with the indicated range of concentrations of either T_3 or GC-1. Transcriptional activation was measured by bioluminescence mediated by luciferase. Data are mean values ± standard deviation from six separate experiments.

and the linkage in thyroid hormone is severely sterically hindered by the two iodine atoms at the 3 and 5 positions. Thus, it soon became apparent to us that a new thyronine scaffold that had the same basic biological properties of T_3 but was more synthetically accessible would be highly desirable.

The X-ray crystallographic structure of the T_3–TR complex reveals a unique internal binding mode for the hormone and indicates that there is a very tight fit between the hormone and LBD [7]. This suggested that an effective thyromimetic must at least be a close mimic of T_3 in terms of overall molecular shape. Drawing on structure–activity relations for thyroid hormone analogs, we designed GC-1 to be a halogen-free thyromimetic. The key features of GC-1 include the replacement of all iodine atoms with methyl and isopropyl groups, replacement of the biaryl-ether linkage with a methylene linkage, and replacement of the amino-acid sidechain with an oxyacetic-acid sidechain. We envisioned that these changes would solve the major synthetic problems outlined above and that GC-1 would be a versatile starting point for the design and synthesis of structurally diverse thyromimetic ligands.

GC-1 was synthesized by a convergent synthetic route wherein the key step is an efficient aldehyde addition reaction that connects the two aryl rings. The only problematic step encountered in the process is the final alkylation procedure of bis-phenol **8**, which does not proceed with the desired selectivity. This problem can be remedied by differentiating the two phenols early in the route with the appropriate protecting groups. The synthetic route is adaptable to analog design. For example, different alkylation reagents could be used in the final step, addition reactions to the carbon bridge could be performed, and different ring substituents could be included.

We were surprised to find that GC-1 has a remarkably high affinity for the TR. The ligand binds to $TR\beta_1$ with the same affinity as T_3 and binds to $TR\alpha_1$ with tenfold lower affinity. The high affinity of GC-1 is somewhat surprising because the analog DIMIT, which contains the 3,5-dimethyl-3′-isopropyl ring pattern, binds to the TR with only 0.01 the affinity of T_3. This suggests that one or both of the other two changes in GC-1, namely the methylene

linkage and/or the oxyacetic-acid sidechain, confers higher affinity than the thyronine structure, and therefore compensates for the deleterious iodine replacements. The methylene bridge analog of T_3 has been made and it shows a modest twofold increase in affinity compared to T_3 [17,18]. It is therefore likely that the oxyacetic-acid sidechain is the group that is responsible for most of the affinity compensation in GC-1, but unequivocal assignment will only be possible after the appropriate T_3 analogs are made and tested. A similar apparent high TR affinity has been reported for the halogen-free thyromimetic **2** (Figure 1), although crude nuclear extract rather than purified TR was used in those experiments [15].

The finding that GC-1 is selective for TRβ over TRα was also surprising. This TRβ selectivity is seen, not only in binding experiments, but also in dose-response cellular transactivation experiments. The thyromimetic **2** (Figure 1) also shows selectivity for TRβ in cellular transactivation assays [16]. Thus, these studies indicate that synthetic ligands can have the property of selectivity for binding and activation of the different TR isoforms whereas thyroid hormone shows no such selectivity. These ligands may be useful for animal studies aimed at understanding the relative roles of TRα and TRβ in mediating the actions of thyroid hormones, and for human studies to determine if use of selective compounds can retain beneficial actions of thyroid hormones with decreased deleterious actions.

Significance

We have designed and synthesized a thyroid hormone receptor (TR) ligand representative of a new class of thyromimetics that have several synthetic advantages over traditional thyromimetics. By changing the biaryl-ether linkage present in thyroid hormone to a methylene linkage we are able to use an efficient C–C bond forming reaction in the key biaryl-coupling step. This process is likely to be more tolerant to different substituents than the analogous biaryl ether coupling reactions suggesting that a greater variety of thyromimetic structural variants will be accessible through this synthetic strategy. The lead compound GC-1 has interesting and potentially useful thyromimetic properties. GC-1 is a high affinity ligand for the TR and shows subtype selectivity for TRβ over TRα in both receptor-binding and ligand-activation functions. Thyroid hormone T_3 does not show such selectivity and binds both TRs with the same affinity. This TRβ selectivity property of GC-1 suggests that GC-1 will be a useful chemical probe to study the physiological role of the different TR subtypes and isoforms *in vivo*.

Materials and methods

Synthesis

2-Isopropyl anisole was prepared using the familiar Williamson-type condensation between an alcohol and a halide [19]. Briefly, a mixture of 2-isopropylphenol (12.0 g, 88.1 mmol), methyl iodide (25.0 g, 176.2 mmol), and potassium carbonate (24.3 g, 176.2 mmol) in 44 ml of dimethyl formamide (DMF) was stirred for 20 h at room temperature. The reaction mixture was diluted with 300 ml of ether and washed with 250 ml of water and 5 × 100 ml of brine. The organic portion was dried ($MgSO_4$), filtered, and evaporated to give an oil, which was purified by flash column chromatography (silica gel, 90:10 hexane/ethyl acetate) to give the pure product (12.5 g, 82.1 mmol, 93%); ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.21 (d, 6H, J = 6.9 Hz), 3.32 (heptet, 1H, J = 6.9 Hz), 3.83 (s, 3H), 6.85 (d, 1H, J = 8.1 Hz), 6.93 (t, 1H, J = 7.5 Hz), 7.17 (t, 1H, J = 8.1 Hz), 7.21 (d, 1H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$ 300 MHz) δ 22.8, 26.9, 55.4, 110.5, 120.7, 126.1, 126.7, 137.2, 156.9.

4-Bromo-2-isopropyl anisole (**3**). To a suspension of KBr (18.8 g, 157.7 mmol) in 400 ml of methylene chloride at 0°C were added 18-Crown-6 (2.08 g, 7.88 mmol), 3-chloroperoxy benzoic acid (27.2 g, 157.7 mmol) and 2-isopropyl anisole (12.0 g, 78.8 mmol). After stirring for 3 h at 0°C, the reaction mixture was poured into ice water (500 ml), and stirred 0.5 h. The organic layer was separated, washed with sat. $NaHCO_3$ solution (400 ml) and then water (300 ml), and dried ($MgSO_4$). The solvent was evaporated to give an oil, which was purified by flash column chromatography (silica gel, 98:2 hexane/ethyl acetate) to give 13 g (56.7 mmol, 72%) of **3** as an oil; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.2 (d, 6H, J = 6.9 Hz), 3.27 (heptet, 1H, J = 6.9 Hz), 3.8 (s, 3H), 6.7 (d, 1H, J = 8.4 Hz), 7.23 (d, 1H, J = 2.4 Hz), 7.26 (dd, 1H, J = 2.4, 8.4 Hz). ^{13}C NMR ($CDCl_3$ 300 MHz) δ 22.6, 26.9, 55.7, 112.2, 113.2, 129.3, 139.6, 156.1. HR–MS, calc'd for $C_{10}H_{13}OBr$, 230.0129; found, 230.0126.

4-Bromo-3,5-dimethyl anisole (**4**). A mixture of 4-bromo-3,5-dimethyl phenol (25.0 g, 124.3 mmol), methyl iodide (35.3 g, 248.6 mmol), and potassium carbonate (34.4 g, 248.6 mmol) in 62.5 ml of DMF was stirred for 2 h at room temperature. The reaction mixture was diluted with 300 ml of ether and washed with 250 ml of water and 5 × 100 ml of brine. The organic portion was dried ($MgSO_4$), filtered, and evaporated to give an oil, which was purified by flash column chromatography (silica gel, 90:10 hexane/ethyl acetate) to give **4** (26 g, 120.8 mmol, 97%); ^{1}H NMR ($CDCl_3$ 300 MHz) δ 2.37 (s, 6H), 3.74 (s, 3H), 6.63 (s, 2H). ^{13}C NMR ($CDCl_3$ 300 MHz) δ 24.2, 55.4, 113.9, 118.4, 139.2, 158.1. HR–MS, calc'd for $C_9H_{11}OBr$, 215.9973; found, 215.9979.

2,6-Dimethyl-4-methoxy benzaldehyde (**5**). To **4** (20 g, 93.0 mmol) in 500 ml of tetrahydrofuran at −78°C was added 120 ml of *tert*-butyllithium (1.7 M in pentane). The reaction mixture was stirred for 30 min at −78°C and then DMF (136.0 g, 186.0 mmol) was added. The reaction mixture was stirred for 1 h at −78°C and for 1.5 h at room temperature, diluted with 300 ml of ether, and washed with 300 ml of water, acidified with 1 N HCl, and 5 × 100 ml of brine. The organic portion was dried ($MgSO_4$), filtered, and evaporated to give the crude product, which was purified by flash column chromatography (silica gel, 90:10 hexane/ethyl acetate) to yield **5** (9.50 g, 57.8 mmol, 62%) as a white solid; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 2.61 (s, 6H), 3.83 (s, 3H), 6.6 (s, 2H), 10.5 (s, 1H). ^{13}C NMR ($CDCl_3$ 300 MHz): δ 21.3, 55.4, 114.8, 126.1, 144.7, 162.9, 191.3. HR–MS, calc'd for $C_{10}H_{12}O_2$, 164.0837; found, 164.0832.

3,5-Dimethyl-4-(3'-isopropyl-4'-methoxybenzylhydroxy) anisole (**6**). To **3** (12 g, 52.4 mmol) in 300 ml of tetrahydrofuran at −78°C was added 68 ml of *tert*-butyllithium (1.7 M in pentane). The reaction mixture was stirred for 10 min at −78°C and then **5** (8.6 g, 52.4 mmol) was added. The reaction mixture was stirred for 1 h at −78°C and for 1.5 h at room temperature, diluted with 150 ml of ether, washed with 150 ml of water, acidified with 1 N HCl, and 5 × 50 ml of brine. The organic portion was dried ($MgSO_4$), filtered, and evaporated to give the crude product, which was purified by flash column chromatography (silica gel, 95:5 hexane/ethyl acetate) to yield **6** (12 g, 38.2 mmol, 73%) as an oil; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.18 (dd, 6H, J = 6.6, 6.9 Hz), 2.25 (s, 6H), 3.29 (heptet, 1H, J = 6.9 Hz), 3.79 (s, 6H), 6.25 (s, 1H), 6.58 (s, 2H), 6.73 (d, 1H, J = 8.7 Hz), 6.89 (dd, 1H, J = 2.1, 8.7 Hz), 7.23 (d, 1H, J = 2.1 Hz).^{13}C NMR ($CDCl_3$ 300 MHz): δ 21.2, 23.8, 27.1, 55.3, 55.7, 71.1, 110.1, 114.5, 123.7, 132.2, 135.1, 136.8, 138.9, 155.7, 158.5. HR–MS, calc'd for $C_{20}H_{26}O_3$, 314.1882; found, 314.1871.

*3,5-Dimethyl-4-(3'-isopropyl-4'-methoxybenzyl) anisole (**7**).* A solution of **6** (2.0 g, 6.36 mmol) in 22 ml of 9% (v/v) AcOH in EtOH containing 10% Pd/C (200 mg) was hydrogenated at 1 atm at room temperature. When hydrogen uptake was complete (12 h), the catalyst was filtered off and the filtrate was diluted with 200 ml of ether, washed with sat. $NaHCO_3$ solution (3 × 50 ml), water (150 ml) and brine (3 × 50 ml). The solvent was evaporated to yield 1.5 g (5.03 mmol, 79%) of **7** as an oil. This material was used in the next step without further purification; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.16 (d, 6H, J = 6.9 Hz), 2.22 (s, 6H), 3.26 (heptet, 1H, J = 6.9 Hz), 3.77 (s, 3H), 3.79 (s, 3H), 3.91 (s, 2H), 6.62 (s, 2H), 6.65-6.70 (m, 2H), 6.95 (d, 1H, J = 2.1 Hz). ^{13}C NMR ($CDCl_3$ 300 MHz): δ 20.7, 22.9, 27.1, 33.9, 55.8, 56.2, 110.4, 113.5, 125.2, 126.1, 129.9, 132.1, 136.9, 138.6, 155.1, 157.7. HR–MS, calc'd for $C_{20}H_{26}O_2$, 298.1933; found, 298.1931.

*3,5-Dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl) phenol (**8**).* To **7** (1.3 g, 4.35 mmol) in 75 ml of methylene chloride at −78°C was added 44 ml of boron tribromide (1.0 M in methylene chloride). The reaction mixture was stirred for 30 min at −78°C and for 10 h at room temperature. The reaction mixture was washed with water (2 × 100 ml), dried ($MgSO_4$), and evaporated to give crude product (1.5 g). Purification using flash column chromatography (silica gel, 80:20 hexane/ethyl acetate) gave **8** (812 mg, 3.00 mmol, 69%); ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.2 (d, 6H, J = 6.9 Hz), 2.18 (s, 6H), 3.15 (heptet, 1H, J = 6.9 Hz), 3.89 (s, 2H), 6.57 (m, 4H), 6.91 (s, 1H). ^{13}C NMR ($CDCl_3$ 300 MHz): δ 20.5, 22.7, 27.3, 33.8, 114.9, 115.3, 126.7, 129.9, 132.5, 134.7, 138.9, 150.9, 153.5. HR–MS, calc'd for $C_{18}H_{22}O_2$, 270.1618; found, 270.1620.

[3,5-Dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl) phenoxy] acetic acid (GC-1). To cesium carbonate (3.01 g, 9.24 mmol) and **8** (500 mg, 1.85 mmol) in 37.5 ml of 37% (v/v) DMF in tetrahydrofuran at −25°C was added *tert*-butylchloroacetate (278.6 mg, 1.85 mmol). The reaction mixture was stirred for 1 h at −25°C and for 30 min at room temperature, poured into 100 ml of cold 1 N HCl, and extracted with ethyl acetate (3 × 150 ml). The combined organic portions were dried ($MgSO_4$) and evaporated to yield 700 mg of crude, which was purified using flash column chromatography (silica gel, 90:10 hexane/ethyl acetate) to yield the product (250 mg), used directly in the following reaction; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 1.2 (d, 6H, J = 6.9 Hz), 1.5 (s, 9H), 2.17 (s, 6H), 3.16 (heptet, 1H, J = 6.9 Hz), 3.88 (s, 2H), 4.50 (s, 2H), 6.55-6.58 (m, 2H), 6.60 (s, 2H), 6.90 (s, 1H). To the above ester (200 mg, 0.520 mmol) in 4 ml of methanol was added 2.6 ml of 1 N NaOH. The reaction mixture was stirred for 1 h at room temperature, acidified with 3 ml of 2 N HCl, and extracted with ethyl acetate (2 × 25 ml). The combined organic portions were dried ($MgSO_4$) and evaporated to give GC-1 (170 mg, 0.518 mmol, 28%); ^{1}H NMR (CD_3OD 300 MHz) δ 1.15 (d, 6H, J = 6.6 Hz), 2.18 (s, 6H), 3.21 (heptet, 1H, J = 6.6 Hz), 3.86 (s, 2H), 4.40 (s, 2H), 6.49-6.62 (m, 2H), 6.65 (s, 2H), 6.84 (s, 1H). ^{13}C NMR (CD_3OD 300 MHz): δ 20.8, 23.0, 27.6, 34.4, 68.3, 115.2, 115.6, 126.0, 126.4, 126.5, 131.6, 135.6, 139.1, 152.8, 157.0, 177.9. HR–MS, calc'd for $C_{20}H_{24}O_4$, 328.1675; found, 328.1679.

Thyroid hormone receptor ligand binding assays

Full-length hTRα_1 and hTRβ_1 were produced using the TNT coupled reticulocyte lysate system (Promega). The ligand binding domains of hTRα_1 and hTRβ_1 were expressed in *Escherichia coli* and purified to greater than 95% homogeneity as described previously [21]. Competition assays for binding of unlabelled T_3, DIMIT, and GC-1 were performed using 1 nM [^{125}I]T_3 in gel filtration binding assays as described [21]. The K_D and standard error (SE) values were calculated by fitting the competition data to the equations of Swillens [22] using the GraphPad Prism computer program (GraphPad Software, Inc.).

Cell culture, transfections and reporter gene assays

GC and HeLa cells were maintained and subcultured in DME H-21, 4.5 g/l Glucose media containing 10% newborn bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transfection procedures were described previously [23,24]. Briefly, cells were collected and resuspended in Dulbecco's PBS (0.5 ml/transfection) containing 0.1% dextrose, 10 μg/ml biobrene, and mixed with 0.5 μg of the appropriate TR expression vector and 10 μg of the reporter plasmid. The TR expression vector contained either the full length human TRβ_1 or human TRα_1 under the control of the cytomegalovirus promoter, CMVTRβ_1 and CMVTRα_1, respectively. The reporter plasmid contained a synthetic TR response element (DR-4) containing two copies of a direct repeat spaced by four nucleotides (AGGTCA-cagg-AGGTCA) cloned immediately upstream of a minimal (−32/+45) thymidine kinase (tk) promoter linked to either chloramphenicol acetyl transferase (CAT) or luciferase coding sequences. Cells (2.0×10^7 or 0.8×10^7, for GC and HeLa cells, respectively) were electroporated using a Bio-Rad gene pulser at 330 (GC cells) or 350V (HeLa cells) and 960 microfarads, pooled in growth medium (DME H-21 with 10% charcoal-treated, hormone-stripped, newborn bovine serum), plated in 6-well dishes, and treated with either vehicle (ethanol), hormone (T_3), or GC-1. T_3 and GC-1 were used at increasing concentrations, 10^{-10}–10^{-7} M and 10^{-11}–10^{-5} M, respectively (for CAT assays, ligand concentrations of 10^{-11}–10^{-7} M and 10^{-11}–10^{-6} M were used for T_3 and GC-1, respectively). After incubation for 24 h at 37°C cells were detached with 1 ml of calcium-magnesium-free PBS, 1 mM EDTA, prewarmed at 37°C, and transferred to 1.5 ml Eppendorf tubes. Cells were pelleted by centrifugation in a microfuge for 1 min at room temperature (RT). The supernatants were aspirated and the pellets were lysed by addition of 120 μl of Tris–Cl 0.25 M pH 7.6, 0.1% Triton. After resuspension by vortexing for 5–10 s, the lysates were pelleted by centrifugation in a microfuge for 5 min at RT. Cellular lysates (75 μl) were then assayed for CAT as previously described [25]. For luciferase activity measurements, 100 μl of the cellular lysate was added to 300 μl of 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM potassium phosphate pH 7.8, 1 mM DTT, 2 mM ATP, 0.2 mM Luciferin [26]. The light output was measured for 10 s at RT with a luminometer (Analytical Luminescence Laboratory, MONOLIGHT^R 1500).

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