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Hammett Analysis of Selective Thyroid Hormone Receptor Modulators Reveals Structural and Electronic Requirements for Hormone Antagonists

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Abstract: Selective thyroid hormone modulators that function as isoform-selective agonists or antagonists of the thyroid hormone receptors (TRs) might be therapeutically useful in diseases associated with aberrant hormone signaling. The most potent thyroid hormone antagonist reported to date is **NH-3**. To explore the significance of the 5′-*p*-nitroaryl moiety of **NH-3** and understand what chemical features are important to confer antagonism, we sought to expand the structure−activity relationship data for the class of 5′-phenylethynyl **GC-1** derivatives. Herein, we describe an improved synthetic route utilizing palladium-catalyzed chemistry for efficient access to a series of 5′-phenylethynyl compounds with varying size and electronic properties. We prepared and tested sixteen analogues for TR binding and transactivation activity. Substitution at the 5′-position decreased binding affinity, but retained TRβ-selectivity. In transactivation assays, the analogues displayed a spectrum of agonist, antagonist, and mixed agonist/antagonist activity that correlated with electronic character in a Hammett analysis between σ substituent value and TR modulation. Analogues **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** displayed full antagonist activity with reduced potency compared to **NH-3**, indicating the nitro group is not required for antagonism. However, para-substitution with strong electron withdrawing properties on the 5′-aryl extension is important for antagonist activity, and antagonist potency—but not ligand receptor binding—was found to correlate linearly with the sigma values for the electron withdrawing substituents.

Introduction

Thyroid hormones regulate a multitude of physiologic effects ranging from embryonic development to maintenance of homeostasis in adults.[1−4] Thyroxine (T_4) is the major form released by the thyroid gland (Figure 1). A lesser amount of 3,5,3′-triiodothyronine (T_3) is also released, but the bulk amount is produced by deiodination of T_4 to T_3 in peripheral tissues. T_3 appears to be the major active form of the hormone in thyroid target tissues. Its action is primarily mediated through the nuclear thyroid hormone receptors (TRs) that regulate transcription of target genes either positively or negatively in response to hormone binding. There are two TR subtypes (α and β) encoded on separate genes, each having two additional isoforms (TRα_1, TRα_2, TRβ_1, TRβ_2) due to differential splicing.[5] Although most

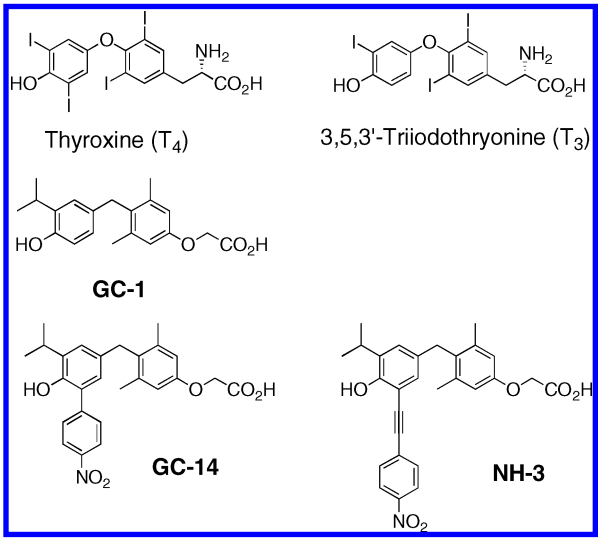

Figure 1. Structures of ligands for the thyroid hormone receptors (TR). T_4 and T_3 are natural thyroid hormones. **GC-1** is a thyromimetic while **GC-14** and **NH-3** are antagonists.

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of these isoforms are widely expressed, there are distinct patterns of expression that vary with tissue and developmental stage. In particular, TRβ_2 is almost exclusively expressed in the hypothalamus, anterior pituitary, and developing ear. Genes positively regulated by T_3 contain a cis-acting thyroid hormone response element (TRE) upstream of the promoter region. Unliganded TRs are bound to the TRE, typically as heterodimers with the retinoid X receptor (RXR), and are associated with a group of corepressor proteins to repress the basal transcriptional machinery. Binding of hormone induces TR release of corepressors and subsequent recruitment of coactivator proteins to enhance TRE-driven transcriptional activity.[6]

The crystal structures of several nuclear receptor (NR) ligand binding domains (LBDs) in the unliganded and liganded (agonist or antagonist) states suggest a common mode of ligand-regulated activation and inhibition of the nuclear receptor superfamily.[7,8] Binding of an agonist ligand induces rearrangement of the LBD, most dramatically in the C-terminal helix 12 (H12). H12 acts as a lid over the ligand binding pocket and contributes to the formation of a hydrophobic cleft at the receptor surface accessible for coactivator binding.[9−11] Conversely, binding of an antagonist ligand induces an inactive receptor conformation by preventing proper packing of H12 to complete the coactivator binding cleft.[12] The crystal structures of the TR LBD bound to agonists such as T_3 and **GC-1** have been solved.[13,14] However, no structure of the TR LBD bound to an antagonist is currently available. Development of potent T_3 antagonists is still in its infancy. To date, most reported T_3 antagonists have moderate to weak potency with IC_{50} values in the high nanomolar to micromolar range in cell culture assays, thereby limiting their characterization in animal models and potential therapeutic utility.[15−19] However, the T_3 antagonist **NH-3** was the first to demonstrate potent inhibition of T_3 action in both cell culture and whole animal-based assays.[1,20]

We have previously shown that the rigid ethynyl moiety of **NH-3** gave improved antagonist efficacy and potency compared to the parent compound **GC-14**.[1,19] However, the contribution of the 5′-*p*-nitroaryl group to antagonist activity is unknown. To better understand the molecular basis of the antagonistic activity of **NH-3** and the significance of the 5′-*p*-nitroaryl pharmacophore, we sought to expand the structure−activity relationship (SAR) data for the 5′-phenylethynyl series of **GC-1** derivatives. We hypothesize that the nitro group is not required for antagonism and that the electronic nature of the 5′-aryl extension will dictate a spectrum of agonist versus antagonist activity. That is, extensions containing electron-donating groups (EDG) will productively interact with receptor residues to stabilize an active receptor conformation, while extensions containing electron-withdrawing groups (EWG) will stabilize an inactive receptor conformation. Herein, we describe the synthesis of sixteen 5′-phenylethynyl **GC-1** derivatives having variable electronic properties. We show that the analogues bind TR with moderate nanomolar affinity and TRβ selectivity, exhibit selective TR modulation that indeed correlates with electronic character, and function similar to **NH-3** with respect to ligand-induced TR interaction with coactivators and corepressors to neutralize TR transcriptional activity.

Results

Chemical Synthesis. The synthesis previously described for the 5′-phenylethynyl **GC-1** derivatives using the palladium-catalyzed Suzuki−Miyaura coupling[1] was modified to improve the 5′-iodination reaction to generate the key intermediate **4** (Scheme 1A). The starting **GC-1** biarylmethane intermediate **1** was treated with tetrabutylammonium fluoride followed by alkylation with methyl 2-bromoacetate to generate the 1-oxyacetic acid side chain protected as the methyl ester **2**. Acidic hydrolysis of the methoxymethyl (MOM)-protected phenol allowed efficient iodination at the 5′-position with iodine monochloride. Reprotection of the phenol **3** as the methoxymethyl ether gave intermediate **4**, with an overall yield of 51% from **1**. This new route allowed preparation of **4** in multigram quantities and offered more efficient access to 5′-phenylethynyl compounds.

Subsequent palladium-catalyzed Suzuki−Miyaura coupling[21] of **4** with phenylethynyl boronate derivatives, generated in situ under basic conditions with MeO-9-BBN, produced 5′-phenylethynyl analogues in good yields (Scheme 1B). Many of the starting phenylacetylenic compounds are commercially available; others were generated via Sonogashira[22] or Sandmeyer[23,24]

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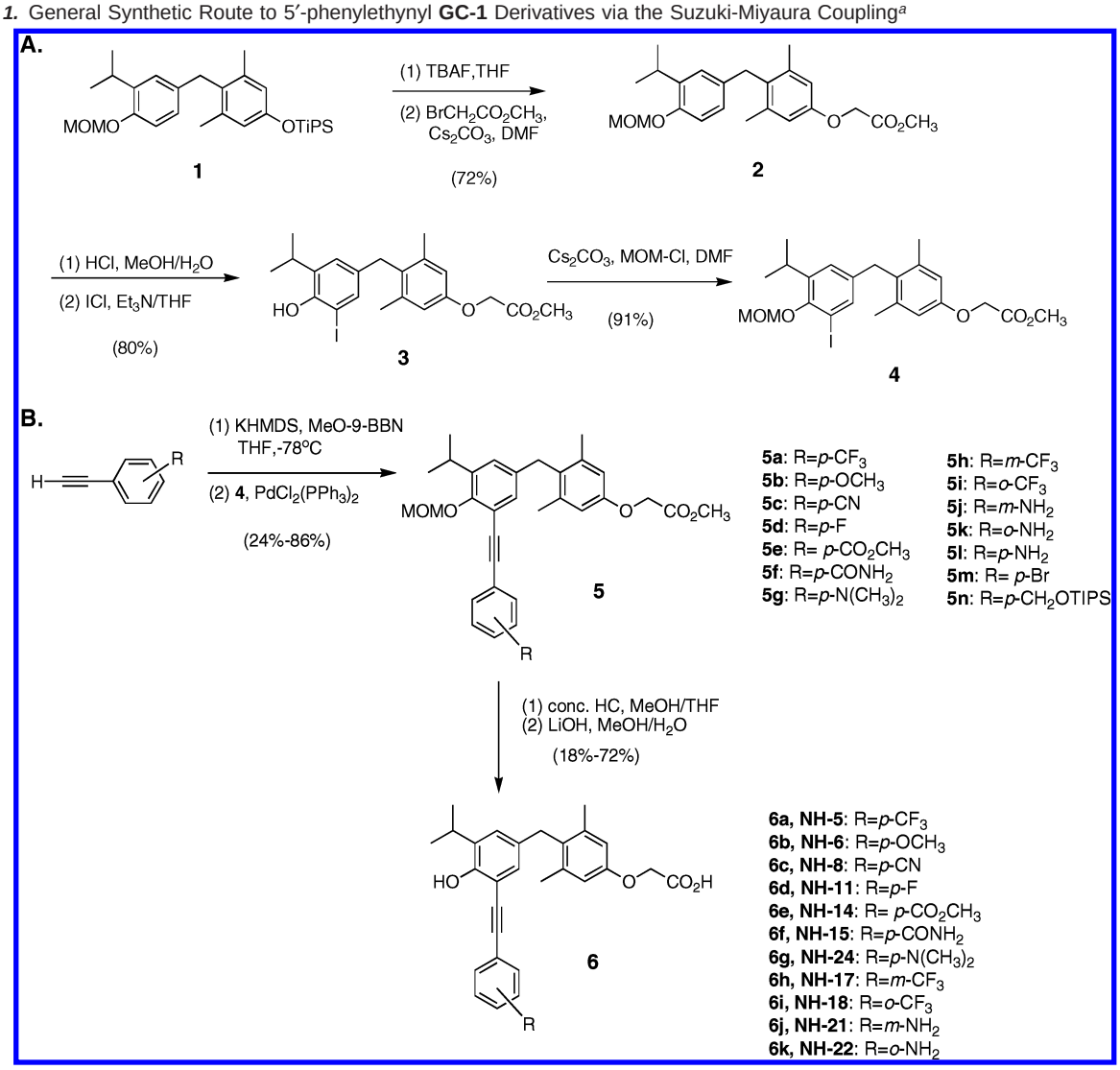
Scheme 1. General Synthetic Route to 5′-phenylethynyl **GC-1** Derivatives via the Suzuki-Miyaura Coupling[a]



[a] (A) Improved synthesis of the 5′-iodinated key intermediate **4**. (B) Suzuki-Miyaura coupling of **4** with phenylacetylenic derivatives followed by deprotection afforded the desired compounds **6a**−**k**. Most phenylacetylenic derivatives were commercially available; others were readily synthesized via standard procedures as described in the Supporting Information.

conditions. The resulting coupled products **5a**−**k** were then subjected to acidic hydrolysis of the methoxymethyl phenolic protecting group followed by basic saponification of the methyl ester to afford the desired 5′-phenylethynyl **GC-1** analogues **6a**−**k** as outlined in Scheme 1B.

Some analogues required additional chemical manipulations after palladium coupling and/or after deprotection steps. The 5′-*p*-azidophenylethynyl analogue **NH-7** was prepared from the aniline precursor **5l** (Scheme 1B) using Sandmeyer[25] conditions of aqueous sodium nitrite in acid and sodium azide (Scheme 2A). Subsequent deprotection of the MOM group and the methyl ester gave the final compound **NH-7** in high yield.

Analogue **NH-16** was obtained by methylation of the 5′-*p*-(dimethylamino)-phenylethynyl intermediate **5g** with methyl trifluoromethanesulfonate in refluxing dichloromethane[26] (Scheme 2B). **NH-16** was not hydrolyzed to the carboxylic acid due to solubility issues in the workup and isolation of the final compound. Analogue **NH-19** was prepared from MOM-deprotected intermediate **7** by oxidation with *m*-chloroperbenzoic acid (*m*-CPBA) in chloroform[27] followed by basic hydrolysis of the methyl ester providing the desired compound in good yield (Scheme 2C). Attempts to oxidize intermediate **5k** (Scheme 1B) to form the 5′-*o*-nitrophenylethynyl analogue unexpectedly resulted in degradation.

The synthesis of analogue **NH-9** started with the coupling of **4** with 1-bromo-4-ethynylbenzene to give intermediate **5m** (Scheme 1B). However, the Suzuki-Miyaura coupling did not go to completion and the coupled product was an inseparable mixture with the aryl iodide **4**. This mixture was carried through another palladium-catalyzed coupling for the synthesis of nitroalkane **8**, adopting the procedure described by Vogl and Buchwald[28] (Scheme 3A). The reaction yield for this coupling was low with multiple byproducts, which likely was due to use of impure bromide **5m**. The MOM protecting group was

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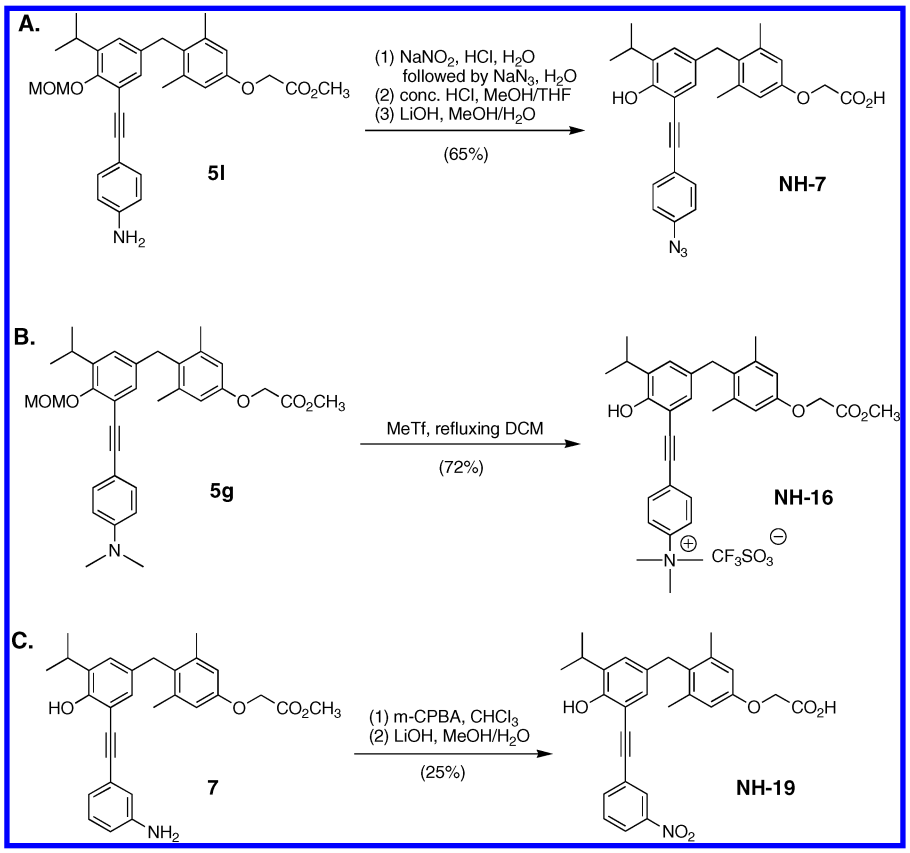
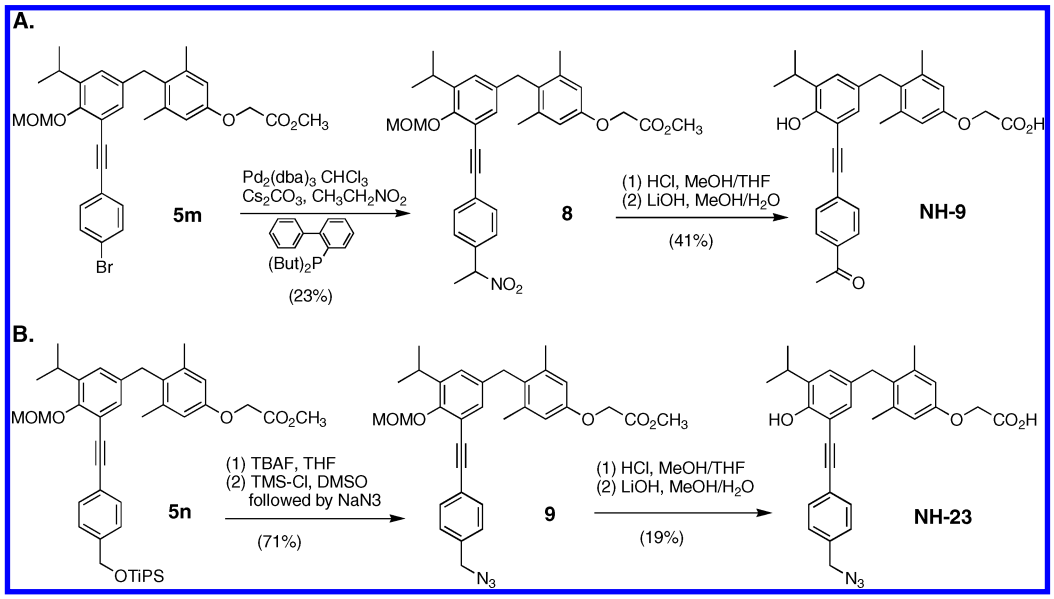
Scheme 2. Synthesis of **NH-7**, **NH-16**, and **NH-19**



Scheme 3. Synthesis of **NH-9** and **NH-23**



removed under acidic conditions. Upon treatment with aqueous lithium hydroxide in methanol for deprotection of the methyl ester, the *p*-nitroethane group underwent hydrolysis in a Nef reaction[29] after acidic workup to give the ketone product **NH-9** (Scheme 3A).

Analogue **NH-23** was synthesized from the triisopropylsilyl (TiPs) protected benzyl alcohol **5n** (Scheme 1B) as outlined in Scheme 3B. De-silylation with tetrabutylammonium fluoride generated the free benzyl alcohol, which was readily converted to the benzyl azide **9** via formation of the benzyl chloride in a one-pot synthesis with chlorotrimethylsilane in DMSO[30] and sodium azide.[31] Subsequent cleavage of the MOM group and the methyl ester afforded **NH-23**.

Under the basic conditions of the final saponification step, a number of 5′-phenylethynyl derivatives underwent cyclization of the *o*-alkynylphenolic moiety to form the benzofuran product.

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Table 1. Binding and Transcriptional Activation Data of 5′-phenylethynyl Derivatives at Human TRα_1 and TRβ_1

5'-substitution	$K_D \pm$ SE (nM)[a]		% TRβ_1 activation[b]	TRβ_1 EC_{50} (IC50) (nM)[c]	% TRα_1 activation[b]	TRα_1 EC_{50} (IC50) (nM)[c]	Activity
	hTRβ_1	hTRα_1					
T_3	0.10 ± 0.03	0.10 ± 0.03	100	2	100	2	Full Agonist
GC-1[d] H	0.10 ± 0.02	1.8 ± 0.2	100	7	100	45	Full Agonist
NH-1	37 ± 9	490 ± 100	70	500	n.d.[e]	n.d.[e]	Partial Agonist
NH-2[f]	0.52 ± 0.05	5.2 ± 0.7	85	380	n.d.[e]	n.d.[e]	Partial Agonist
NH-3[g]	2.8 ± 0.4	14 ± 3	3	(230)	5	(1200)	Full Antagonist
NH-4	90 ± 6	330 ± 60	2	n.d.[h]	n.d.	n.d.	-
NH-5	0.97 ± 0.05	8.4 ± 0.1	6	(590)	1	(1600)	Full Antagonist
NH-6[f]	1.7 ± 0.2	13.6 ± 0.3	75	99	n.d.[e]	n.d.[e]	Partial Agonist
NH-7	2.5 ± 0.4	23.7 ± 0.5	5	(630)	1	(2600)	Full Antagonist
NH-8[f]	3.1 ± 0.5	17.0 ± 0.4	40	750 (>2000)	n.d.	n.d.	Mixed Agonist/ Antagonist
NH-9	3.1 ± 0.5	23.0 ± 0.5	2	(1700)	1	(>5000)	Full Antagonist
NH-11	12 ± 2	46 ± 13	1	(4200)	2	(>5000)	Full Antagonist
NH-14[f]	3 ± 1	38 ± 5	37	350 (>3000)	n.d.	n.d.	Mixed Agonist/ Antagonist
NH-15	10 ± 1	41 ± 6	1	n.d.[h]	n.d.	n.d.	-
NH-16	453 ± 30	629 ± 32	58	2700	n.d.[e]	n.d.[e]	Partial Agonist
NH-24	0.23 ± 0.02	3.9 ± 0.6	57	180	63	280	Partial Agonist
NH-23	0.68 ± 0.04	8.1 ± 0.8	2	(1100)	1	(>5000)	Full Antagonist
NH-17	57 ± 22	332 ± 16	1	(>2500)	n.d.	n.d.	Weak Antagonist
NH-18	62 ± 14	80 ± 24	1	(>2500)	n.d.	n.d.	Weak Antagonist
NH-19	5 ± 1	31 ± 9	1	(>2500)	n.d.	n.d.	Weak Antagonist
NH-21	11 ± 2	50 ± 14	1	n.d.[h]	n.d.	n.d.	-
NH-22	11 ± 3	67 ± 3	1	n.d.[h]	n.d.	n.d.	-

[a] The K_D and standard error (SE) values are expressed relative to the K_D of T_3 and were calculated by fitting the competition data to the equations of Swillens[32] and using the Graph-Pad Prism computer program (Graph-Pad Software, Inc.). [b] Luciferase activity of 10^{-5} M analogue is expressed as a percent of the TRβ1 or TRα1 response with 10^{-6} M T_3. Values are the mean ± SD for three separate experiments. See Supporting Information for more details. [c] The EC_{50} value is the concentration of ligand required for half-maximum activation, whereas IC_{50} value is the concentration of ligand required for half-maximum inhibition in competition experiments with 2×10^{-9} M T_3. EC_{50} and IC_{50} values were calculated by nonlinear regression with the Graph-Pad Prism computer program (Graph-Pad Software, Inc.) using a sigmoidal dose−response and single-site competition models, respectively. Values reported are the mean for three separate experiments with R^2 fit of at least 0.85 unless value is expressed as greater than (">"), indicating a poor R^2 fit. [d] Refer to Chiellini et al.[19] [e] Compounds exhibiting thyromimetic transcriptional activation through hTRβ1 were not further characterized with hTRα_1. [f] For transactivation assays, HeLa cells were cultured in 10% hormone-depleted, heat-treated (80 °C, 20 min) newborn calf serum during incubation with ligand. Incubation with serum not heat-treated resulted in little or no ligand activity (data not shown), suggesting serum components are significantly sequestering ligand. [g] NH-3 was retested in binding and transactivation assays. Results were comparable to previously reported values of 20 ± 7 nM for hTRβ_1 and 93 ± 29 nM for hTRα_1 in binding, and IC_{50} = 370 nM for hTRβ_1 in antagonist potency. [h] Compound did not induce TR-mediated transactivation at 10^{-5} M nor antagonize 2×10^{-9} M T_3-induced transactivation in a dose-dependent manner. Therefore, EC_{50} and IC_{50} could not be obtained for TRα1 and TRβ1.

The key diagnostic signals in the ^{1}H NMR indicating benzofuran formation were additional peaks near 1.4 ppm (−CH_3, *i*Pr, doublet), near 3.4 ppm (−CH, *i*Pr, heptet), and a peak ranging from 7.2 to 7.8 ppm (vinyl −H, singlet). Cyclization generally occurred at a much slower rate than ester hydrolysis and was limited by controlling the reaction time. Benzofuran formation was also observed for some analogues under slight acidic conditions needed for NMR characterization and consequently limited acquisition of ^{13}C NMR spectra.

TR Binding and Transactivation Properties. All compounds were tested for binding to hTRα_1 and hTRβ_1 by in vitro radioligand displacement assays (see Supporting Information) and the results are summarized in Table 1. K_D values reported are expressed relative to the K_D of T_3 and were calculated by fitting the data to the equations of Swillens.[32] Data of T_3, **GC-**

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1[19] and previously published NH compounds (**NH-1** through **NH-4**)[1] are included for comparison. As observed with other 5′-phenylethynyl analogues having the core **GC-1** scaffold, analogues **NH-5** through **NH-24** retained TRβ-selectivity with impaired affinity compared to **GC-1**. The TRβ-selectivity was consistent with structural and chemical data that suggest the key molecular determinant of selectivity is located on the 1-oxyacetic acid side chain of the **GC-1** core structure.[14,33] The analogues bound hTRβ with low nanomolar affinity with K_D values ranging from 0.5 to 450 nM. Analogue **NH-16** with the masked 1-oxyacetic acid moiety also bound TRβ with reasonable affinity, suggesting the compound might be partially hydrolyzed to the acid form under the conditions of the binding assays. Varying the position of the aryl substituent from para- to ortho- or meta-positions generally led to a decrease in binding affinity. For example, **NH-19** exhibited almost 2-fold decreased binding affinity compared to **NH-3**. Likewise, **NH-17** and **NH-18** had 60-fold lower affinity relative to **NH-5** at TRβ.

The analogues were then tested in human HeLa cells for transcriptional transactivation properties using a luciferase reporter assay. HeLa cells were transiently transfected with expression plasmids for hTRα_1 or hTRβ_1 and a TRE-driven (DR4 element)[34] luciferase reporter as described previously (see Supporting Information).[1] Analogues **NH-6**, **NH-16**, and **NH-24** exhibited partial agonism at TRβ by at least 60% activity relative to saturating T_3-induced transactivation (Figure 2A). The EC_{50} values are reported in Table 1. **NH-16** exhibited similar maximal transactivation activity with reduced potency compared to **NH-24**. We surmise that the apparent agonist activity of **NH-16** resulted from partial demethylation of the trimethylammonium salt and partial hydrolysis of methyl ester to give **NH-24**. Analogues **NH-8** and **NH-14** exhibited mixed agonist and antagonist activity at TRβ. These compounds alone were able to induce luciferase expression by approximately 40% relative to saturating T_3-induced transactivation, while in competition assays they were able to partially block 2 nM T_3-induced activity down to the level of compound alone with micromolar potency (Figure 2B). EC_{50} values were not calculated for **NH-6**, **NH-8**, **NH-14**, and **NH-16** at TRα because their relatively poor affinity and potency for TRα made it difficult to obtain complete dose response curves. Analogues **NH-15**, **NH-21**, and **NH-22** displayed weak activity at TRα and TRβ, as previously observed with **NH-4**. These compounds did not induce TR-mediated transactivation above the level of vehicle control and failed to compete with 2 nM T_3-induced transactivation in a dose dependent manner (Table 1). At 10 μM concentration, **NH-15**, **NH-21**, and **NH-22** exhibited mild antagonism to block T_3 activity by approximately 10% (data not shown). All 5′-phenylethynyl analogues generally became toxic to the cells at doses above 10 μM.

Analogues **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** induced minimal reporter expression above the level of vehicle control with either TRα or TRβ (Figure 2A). In competition assays these compounds exhibited full antagonism in completely blocking 2 nM T_3-induced transactivation in a dose-dependent manner down to the level of activation observed with vehicle alone (Figure 2C). The IC_{50} values for antagonism under these conditions are shown in Table 1. For TRβ, **NH-5** and **NH-7** (IC_{50} = 590 nM and 630 nM, respectively) had 2- to 3-fold reduced potency compared to **NH-3** (IC_{50} = 270 nM). The other antagonists in this group had IC_{50} values in the micromolar range. For TRα, the antagonists exhibited approximately 4- to 5-fold reduced potency compared to TRβ. Thus, these compounds are TRβ-selective in binding affinity as well as transcriptional activity.

Transactivation data for **NH-17**, **NH-18**, and **NH-19** revealed the significance of substitution at the para-position on the 5′-aryl extension for antagonism. The meta- and ortho-substituted analogues induced minimal transactivation above that of vehicle control (Figure 2A). However, these compounds inefficiently blocked 2 nM T_3-induced transactivation and complete dose−response curves could not be obtained. **NH-19** at 10 μM blocked approximately 60% of T_3-induced response at TRβ (data not shown). Similarly, 10 μM of **NH-17** and **NH-18** partially blocked the T_3 response by 50% and 30%, respectively, at TRβ (data not shown). The relative lower affinity of these compounds can partially account for their impaired transactivation activity compared to the para-substituted counterparts **NH-3** and **NH-5**. Combined, these results suggest that substitution at the para-position on the 5′-aryl extension is optimal for ligand binding and antagonist activity.

Affect of Antagonists on TR Interaction with NCoR and GRIP-1. The full antagonists **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** were tested for their affect on TR interaction with coactivator GRIP-1 and corepressor NCoR in mammalian two hybrid assays. HeLa cells were transiently transfected with expression plasmids for the yeast GAL4 DBD linked to either NCoR (*aa*1925−2308) or GRIP-1 (*aa*618−1121), hTRβ-LBD fused to the VP16 activation domain, and a GAL4-driven luciferase reporter (see Supporting Information).[1] As shown in Figure 3A, the 5′-phenylethynyl antagonists failed to stimulate binding of GRIP-1 to TR. The analogues also blocked 10 nM T_3-induced TR-GRIP-1 interactions albeit with weak potency. Increasing antagonist concentration led to decreased T_3-induced TR-coactivator interaction. Dose−response curves could not be obtained due to concentration and toxicity limitations. As with **NH-3**, the inability of these compounds to promote interaction between TR and its target coactivators is one of the underlying mechanisms for their observed antagonist activities.

We then examined the ability of the analogues to promote release of NCoR upon binding to TR. Like T_3 and **GC-1**, the antagonists induced TR−NCoR dissociation in a dose-dependent manner (Figure 3B, 3C). The IC_{50} values of TR-NCoR interaction with bound **GC-1** versus bound 5′-phenylethynyl analogues vary by at least 1 order of magnitude, consistent with the difference in their relative binding affinities for TR. These results suggest that in the presence of **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23**, TR adopts an inactive conformation where neither corepressors nor coactivators are recruited and TR transcriptional activity is nullified. This mechanism of action is not unique to **NH-3** and can be generalized to the class of 5′-para-substituted phenylethynyl derivatives bearing 5′-electron-withdrawing substituents.

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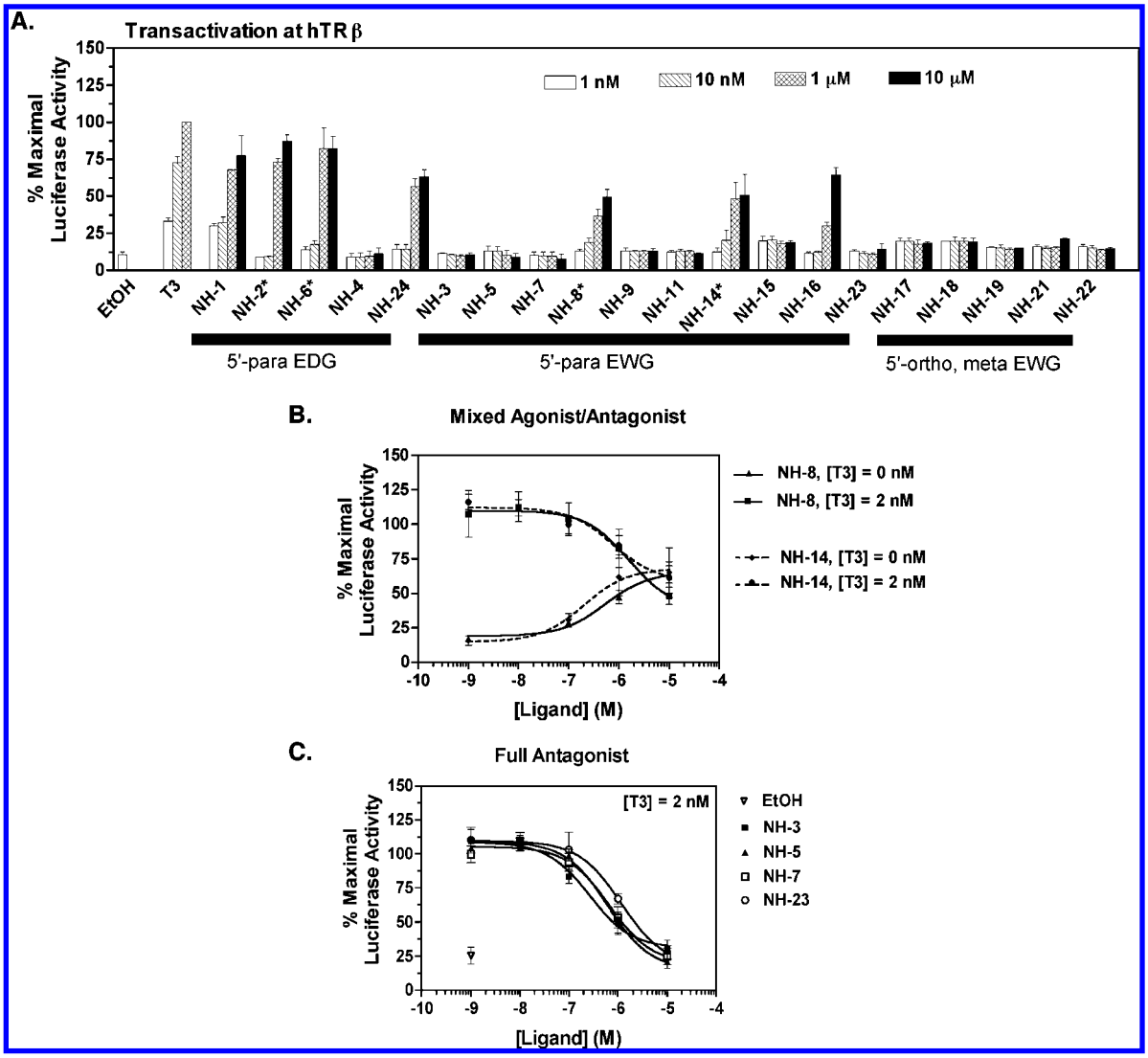

Figure 2. (A) Transcriptional activation of T_3 and 5′-phenylethynyl analogues in luciferase reporter gene assays at hTRβ_1 (see Supporting Information). Analogues with electron donating character generally have agonist activity, while those with electron withdrawing character are nonagonists. [*] indicates data obtained using media supplemented with 10% heat-treated (80°C, 20 min.) newborn calf serum. (B) Dose−response curves of mixed agonist/antagonist **NH-8** and **NH-14** at hTRβ_1 with and without 2 nM T_3. **NH-8** and **NH-14** induced approximately 40% transactivation relative to 2 nM T_3. In competition assays, the compounds blocked T_3-induced transactivation to the level of activation observed for compound alone. (C) Dose−response curves of **NH-3**, **NH-5**, **NH-7**, and **NH-23** at hTRβ_1 in competition with 2 nM T_3. **NH-9** and **NH-11** show similar competition dose−response curves (data not shown). These compounds failed to induce transactivation and completely inhibited the T_3-induced response to the level of ethanol vehicle control. IC_{50} values under these conditions are shown in Table 1. Transactivation induced by 2 nM T_3 is defined as 100% maximal luciferase activity. Values are the mean ± SD for three separate experiments. Dose−response data were fitted by nonlinear regression using the Graph Pad Prism computer program (Graph Pad Software Inc.) for a single site competition model to generate IC_{50} values.

Hammett Analysis. On the basis of the transactivation data of the 5′-para-substituted phenylethynyl derivatives, we generated a Hammett semilog plot of relative ligand potency [log-(IC_{50H}/IC_{50X})] versus the Hammett sigma para (σ_p) substituent values[35,36] (Figure 4A), which were derived from ionization constants in water of para-substituted benzoic acids relative to benzoic acid. These σ_p values incorporate both inductive and resonance contributions to electronic effects. Although extended forms of the Hammett equation incorporating additional parameters such as steric, hydrophobic, and hydrogen-bonding effects would be more accurate to derive quantitative structure−activity relationships (QSAR) for interactions of organic compounds in biological systems, the standard σ_p values are sufficient for a broad qualitative correlation analysis.

A general trend, with the exception of **NH-7**, was observed between the positive σ_p values of substituted aromatics and ligand antagonist activity where greater electron withdrawing character translated to improved antagonist potency (Figure 4A). For example, **NH-5** ($\sigma_p = +0.54$) possessed greater electronic character relative to **NH-11** ($\sigma_p = +0.06$) and exhibited stronger antagonist potency. **NH-3** had the greatest σ_p value ($\sigma_p = +0.78$) for the 5′-phenylethynyl derivatives and remained the most potent TR antagonist reported. Antagonist **NH-23** was not included in the Hammett analysis since no comparable σ_p value could be found in the literature for the benzyl azide substituent. Qualitative Hammett analysis of negative σ_p values and relative ligand potency revealed no significant correlation (data not

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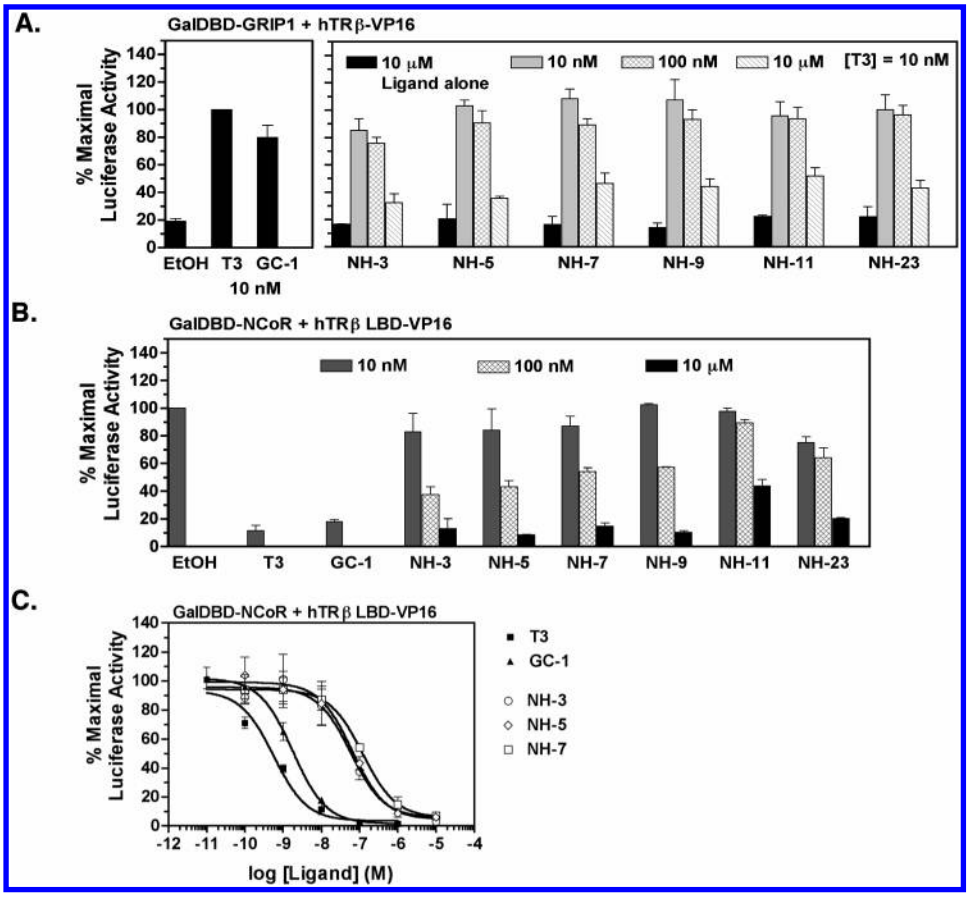

Figure 3. Affect of antagonists **NH-3**, **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** on TR interactions with corepressor and coactivator in mammalian two-hybrid assays (see Experimental section). (A) At 10 μM concentration, the antagonists fail to recruit GRIP-1 to TR. However, increasing antagonist concentration inhibited with micromolar potency the TR-GRIP-1 interactions induced by 10 nM T_3. Competition dose−response curves could not be obtained due to concentration and toxicity limits. TR-GRIP-1 interaction in the presence of 10 nM T_3 is defined as 100% maximal luciferase activity. (B) Like T_3 and **GC-1**, the antagonists were able to promote NCoR dissociation from TR in a dose-dependent manner. (C) **NH-3**, **NH-5**, and **NH-7** inhibited TR-NCoR interaction by 50% (IC_{50}) at 58 nM, 64 nM and 120 nM, respectively. TR-NCoR interaction in the presence of ethanol vehicle control is defined as 100% maximal luciferase activity. All values are expressed as the mean ± SD for three separate experiments. IC_{50} values were calculated using the Graph-Pad computer program (Graph-Pad Software Inc.) by nonlinear regression of a sigmoid dose−response model.

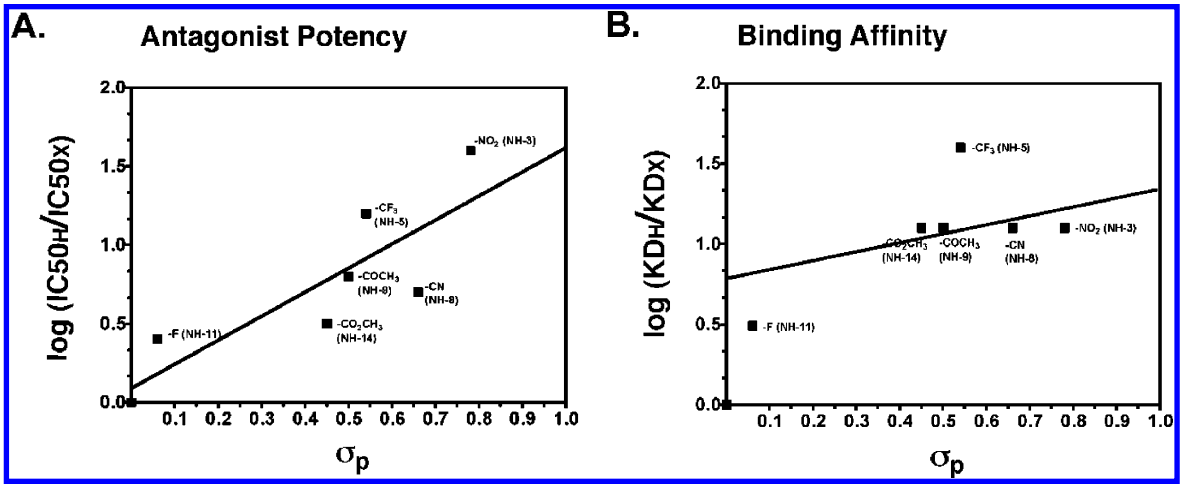

Figure 4. (A) Hammett analysis of electronic character (σ_p) and relative transcriptional activity (log IC_{50H}/IC_{50x}) showed a linear trend ($r^2 = 0.72$, slope = 1.52) of improved antagonist potency with increased electron withdrawing character of the 5′-para-substituted aryl extension. IC_{50H} was assigned a value of 10 μM. (B) Hammett analysis of electronic character (σ_p) and relative binding affinity (log K_{DH}/K_{DX}) revealed essentially no linear correlation ($r^2 = 0.17$, slope = 0.56) between the two parameters. Hammett σ_p substituent values were based on values reported by Hansch et al.[35] and Jaffe:[36] X/σ_p = H/0; F/0.06; N_3/0.08; CO_2CH_3/0.45; $COCH_3$/0.50; CF_3/0.54; CN/0.66; NO_2/0.78.

shown); however, compounds bearing an EDG such as **NH-1**, **NH-2**, **NH-6**, and **NH-24** had partial or full agonist activity.

Similar Hammett analysis of the relative ligand binding affinity [log(K_{DH}/K_{DX})] versus the σ_p parameter revealed no

significant correlation between binding affinity and electronic character of the 5′-aryl extension (Figure 4B). The presence of a strong EDG or EWG did not improve nor impair binding. For example **NH-6** ($\sigma_p = -0.27$) has electron-donating character while **NH-3** ($\sigma_p = +0.78$) and **NH-5** ($\sigma_p = +0.54$) have electron-withdrawing character, yet these compounds had comparable binding affinity and selectivity to TRβ (Table 1). Lack of correlation between binding affinity and electronic properties of the 5′-aryl extension suggests that altering the electronic properties primarily affects TR functional activity through downstream transactivation signaling events.

Discussion

In the present study we evaluated, the significance and role of the 5′-*p*-nitroaryl moiety of **NH-3** for T_3 antagonist activity. We synthesized a series of 5′-phenylethynyl **GC-1** derivatives varying in size and electronic property. All analogues bound TRα and TRβ with moderate nanomolar affinity and 4-fold to 20-fold TRβ selectivity (Table 1). In transactivation assays with TRβ (Figure 2), **NH-6**, **NH-16**, and **NH-24** had partial agonist activity, while **NH-8** and **NH-14** exhibited mixed agonist/antagonist activity. **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** were full antagonists with reduced potency relative to **NH-3**.

We further tested the affect of the antagonists **NH-5**, **NH-7**, **NH-9**, **NH-11**, and **NH-23** on TR interactions with NCoR and GRIP-1 (Figure 3). Similar to **NH-3**, these compounds functioned like agonists to promote corepressor release from TR but failed to induce recruitment of coactivator to TR. Thus, the 5′-phenylethynyl derivatives containing para-substituted EWG are a unique class of selective TR modulators (STRMs) that effectively induce a chemical TR knockout by preventing TR-mediated hormone transactivation and relieving trans-repression of positively- regulated target gene expression.

Closer analysis of the chemical features important for TR modulation by Hammett correlation between TR-mediated ligand activity and electronic character revealed 5′-aryl extensions with EDG (negative σ_p value) had agonist activity while those carrying charge neutral EWG (positive σ_p value) had antagonist activity (Figure 4A). Moreover, greater antagonist potency was observed with greater electron withdrawing character (greater σ_p value) as with **NH-3**, **NH-5**, and **NH-7**. **NH-3** had the greatest σ_p value in this class of compounds and remained the most potent T_3 antagonist. The antagonistic potency of azide **NH-7** did not follow the linear correlation of the other six antagonists for reasons we do not understand presently. The combined SAR data reveal the 5′-*p*-nitroaryl moiety was not required for antagonism. However, substitution with a strong EWG on the 5′-aryl extension was essential for antagonist activity.

Hammett analysis of the ligand binding affinity relative to the σ_p substituent values confirmed that varying the electronic character of the ligand predominantly affects the downstream signaling interactions of TR and not binding. As shown in Figure 4B, there was variability in the binding affinities of the 5′-phenylethynyl derivatives but no correlation was observed between electronic property and changes in binding affinity. Compounds having EDG aryl extensions bound TR with similar affinities as those bearing EWG. Moreover, stronger electronic character does not enhance binding affinity.

Our data further lends support to the observation that the nature and position of the 5′-aryl substituent is critical for T_3 antagonist activity and potency. Analogues with EWG in the ortho- or meta-positions exhibited significantly reduced antagonist potency as seen with analogues **NH-17**, **NH-18**, and **NH-19** compared to the para-substituted counterparts **NH-3** and **NH-5**. This result can partially be accounted for by the reduction in electronic contribution to the aryl extension in the meta- and ortho-positions relative to the para-position.[35] However, compounds with weaker para-electron withdrawing substituents, such as **NH-7** and **NH-23**, also displayed full antagonist activity indicating electronic properties alone do not dictate antagonism. The azido groups of **NH-7** and **NH-23** may be involved in stabilizing an inactive TR conformation.

To better understand the molecular basis of antagonism for this class of STRMs, the 5′-phenylethynyl extension was modeled into the TR ligand binding pocket based on the X-ray crystal structure of **GC-1** bound to hTRβ-LBD.[14,37] Modeling revealed that the 5′-extension clashed with numerous receptor residues in the active conformation, including Phe455 and Phe459 of helix H12. In addition to sterically perturbing proper folding and rearrangement of H12, we postulate that the EWG on the 5′-aryl extension promotes favorable aromatic π-interactions (center-to-edge, edge-to-face, and/or face-to-face)[38] with the phenylalanine residues to stabilize an inactive receptor conformation. Given that the 5′-phenylethynyl **GC-1** antagonists induce a conformation that occluded binding of both coactivators and corepressors, the ligand extension may induce a conformation of H12 that packs against the hydrophobic groove where the coactivator and corepressor binding sites overlap.[10,39,40] This mode of "active antagonism"[41] is similar to that observed for the estrogen receptor (ER) bound to selective ER modulators (SERMs) raloxifene[42] and 4-hydroxytamoxifen,[43] where H12 adopts an auto-inhibitory conformation to compete with coactivator recruitment by mimicking the interactions of the coactivator with the ER-LBD.

In summary, the results of this study confirmed our hypothesis that the 5′-*p*-nitroaryl extension is not required for antagonism and that the size, position, and electronic nature of the 5′-aryl extension will dictate a spectrum of agonist and antagonist activity. A better understanding of the pharmacophore important for T_3 antagonism will be useful for the development of therapeutics for the treatment of diseases associated with excessive thyroid hormone production and action such as hyperthyroidism (thyrotoxicosis).[44] Current therapies for hy-

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perthyroidism usually require weeks before symptoms are relieved or provide only partial relief. Direct blockade of T_3 action with T_3 antagonists would bypass such complications and provide a more effective treatment of hyperthyroidism. Furthermore, TR subtype-selective STRMs will be useful pharmacological probes for studying thyroid hormone signaling pathways.

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Supporting Information Available: Experimental procedures for chemical synthesis of novel compounds and protocols for binding and transactivation assays are included (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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