A Selective TSH Receptor Antagonist Inhibits Stimulation of Thyroid Function in Female Mice

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Because the TSH receptor (TSHR) plays an important role in the pathogenesis of thyroid disease, a TSHR antagonist could be a novel treatment. We attempted to develop a small molecule, drug-like antagonist of TSHR signaling that is selective and active in vivo. We synthesized NCGC00242364 (ANTAG3) by chemical modification of a previously reported TSHR antagonist. We tested its potency, efficacy, and selectivity in a model cell system in vitro by measuring its activity to inhibit stimulation of cAMP production stimulated by TSH, LH, or FSH. We tested the in vivo activity of ANTAG3 by measuring its effects to lower serum free T_4 and thyroid gene expression in female BALB/c mice continuously treated with ANTAG3 for 3 days and given low doses of TRH continuously or stimulated by a single administration of a monoclonal thyroid-stimulating antibody M22. ANTAG3 was selective for TSHR inhibition; half-maximal inhibitory doses were 2.1 μM for TSHR and greater than 30 μM for LH and FSH receptors. In mice treated with TRH, ANTAG3 lowered serum free T_4 by 44% and lowered mRNAs for sodium-iodide cotransporter and thyroperoxidase by 75% and 83%, respectively. In mice given M22, ANTAG3 lowered serum free T_4 by 38% and lowered mRNAs for sodium-iodide cotransporter and thyroperoxidase by 73% and 40%, respectively. In conclusion, we developed a selective TSHR antagonist that is effective in vivo in mice. This is the first report of a small-molecule TSHR antagonist active in vivo and may lead to a drug to treat Graves' disease. **(*Endocrinology* 155: 310–314, 2014)**

The TSH receptor (TSHR) is known to play an important role in the pathogenesis of several thyroid diseases (1). For Graves' disease (GD), especially for Graves' ophthalmopathy, and for thyroid cancer, a TSHR antagonist could be an important new drug treatment. Indeed, in a recent editorial, Emerson (2) asked "(w)hen will TSHR antagonists be available for clinical use?" Two types of TSHR antagonists have been described: anti-TSHR antibodies (for review, see Reference 3) and small-molecule, drug-like compounds (for review, see Reference 4). However, all of the pharmacological studies characterizing small-molecule antagonists have been performed in vitro, and until the present time, they have not been shown to be effective in vivo.

Small-molecule TSHR antagonists have been reported by our group (5–9) and one other group (10, 11). The initial studies of these antagonists were performed in model cell systems made to express human TSHRs (5, 10) or in primary cultures of human thyrocytes (5). More recently, assuming a potentially important use of TSHR antagonists would be to treat Graves' ophthalmopathy, these antagonists have been shown to be effective inhibitors of TSHR activation in fibroblasts/preadipocytes and adipocytes obtained from Graves' orbital tissues (8, 9, 11). In

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Abbreviations: ANTAG3, TSHR antagonist; DIO2, deiodinase type 2; FT4, free T_4; GD, Graves' disease; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; M22, monoclonal thyroid-stimulating antibody; NIS, sodium-iodide cotransporter; TG, thyroglobulin; TPO, thyroperoxidase; TSHR, TSH receptor.

addition to TSH stimulation, these antagonists have been shown to inhibit TSHR stimulation by sera from patients with GD (7) and by a monoclonal thyroid-stimulating antibody (M22) isolated from a patient with GD (9–11). Thus, these compounds have been shown to be effective inhibitors of TSHR activation by all stimuli tested.

In this paper, we report a new analog (NCGC00242364)(ANTAG3) of our previously described antagonist NCGC00161856 that exhibits two properties that are important for a drug to treat humans. This new antagonist appears selective for TSHR because it does not inhibit activation of LH or FSH receptors, the receptors with the highest homology to TSHR within the seven-transmembrane domain (12) in which our small-molecule TSHR antagonists bind (5), and, as shown here, it inhibits TSHR activation in mice in vivo.

Materials and Methods

Synthesis of small-molecule ligand NCGC00242364 (ANTAG3)

The synthetic scheme for the TSHR antagonist ANTAG3 is provided in the Supplementary Information, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Cell culture and cAMP assay

Generation of the cells stably expressing TSHRs, LH receptors, and FSH receptors was described previously (5). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 10 μg/mL streptomycin (Life Technologies Inc) at 37°C in a humidified 5% CO_2 incubator. For measurement of stimulated cAMP production, cells were seeded into 24-well plates at a density of 2.2×10^5 cells/well 24 hours before the experimental incubation. After removal of the growth medium, cells were incubated for 30 minutes in 0.25 mL Hanks' balanced salt solution (HBSS; Cellgro; Mediatech, Inc) with 10 mM HEPES (Cellgro), pH 7.4 and then subsequently in 0.25 mL HBSS/HEPES with the appropriate doses of ANTAG3 (0–30 μM) for 20 minutes. After this preincubation, the medium was replaced with 0.25 mL HBSS/HEPES containing 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich Co) and the EC_{50} dose of the ligand of interest (bovine TSH; Sigma-Aldrich; human LH or human FSH; National Hormone and Peptide Program, Harbor-UCLA Medical Center) and the appropriate doses of ANTAG3 (0–30 μM) in a humidified 5% CO_2 incubator at 37°C. After 60 minutes, the incubation was terminated by adding 0.25 mL lysis buffer (Tropix; Applied Biosystems). The total cAMP content was determined with the Tropix cAMP screen assay. Data were analyzed using GraphPad Prism 5 for Windows (GraphPad Software Inc). The following parameters were applied for the data analysis and curve fitting: nonlinear regression analysis (curve fit), dose response inhibition, and log (inhibitor) vs response (three parameters).

Animal experiments

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Female BALB/c mice (National Cancer Institute Animal Production Program) were maintained on standard rodent chow (NIH-31; Zeigler Brothers, Inc). Mice were group caged up to five and were on a 12-hour light, 12-hour dark cycle (6 AM to 6 PM). Water and feed were available ad libitum. At the time of the experiments, mouse ages ranged from 8 to 13 weeks, with a mean of 10.0 ± 0.12 weeks, and the mean body weight of 18.7 ± 0.13 g. We measured the effects of ANTAG3 in mice on serum free T_4 (FT4) levels and expression of thyroid genes: thyroglobulin (*TG*), deiodinase type 2 (*DIO2*), TSHR, sodium-iodide cotransporter (*NIS*), and thyroperoxidase (*TPO*). Serum FT4 was measured by the GammaCoat free T_4 RIA (DiaSorin S.p.A.) using the manufacturer's protocol. Thyroid expression of mRNAs was measured by quantitative PCR as described elsewhere (13).

In the first series of experiments, we administered TRH (2.4 μg/d; Sigma-Aldrich) without or with ANTAG3 (2 mg/d) by an ALZET osmotic pump (number 1003D, 1 μL/h; DURECT Corp) for 3 days. ANTAG3 was dissolved in formulation number 7, which is composed of the following in percentages (wt/wt): PEG300, 25; glycofurol, 25; cremophor, 25; ethanol, 15; pro-

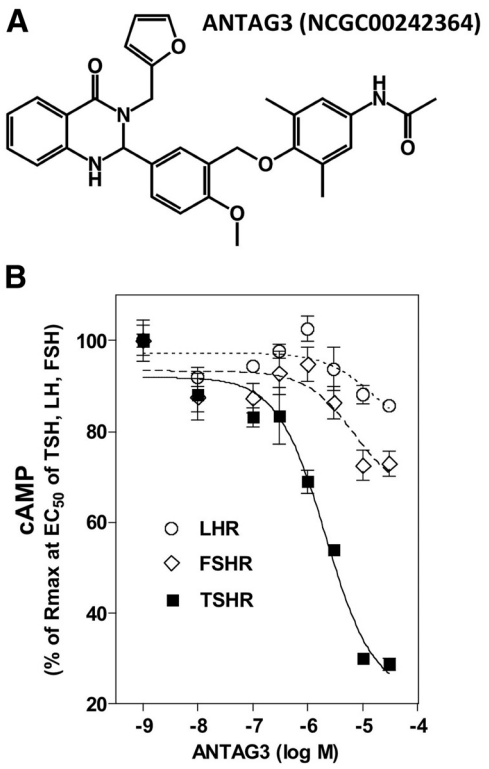

Figure 1. ANTAG3 structure and selectivity. A, Structure of ANTAG3. B, Antagonist activity of ANTAG3 in cells in culture. Human embryonic kidney 293 cells stably expressing TSHRs, LHRs, or FSHRs were preincubated with various doses of ANTAG3 for 20 minutes in HBSS without IBMX. Subsequently the cells were stimulated with the EC_{50} of TSH (1.8 nM), LH (1 nM), or FSH (1 nM) in the presence of varying doses of ANTAG3 in the presence of IBMX. cAMP production was measured after 60 minutes. The results are the mean ± SE in three experiments.

pylene glycol, 10 (14). PEG300 was purchased from Hampton Research. Glycofurol, cremophor, and propylene glycol were purchased from Sigma-Aldrich Co. LLC. Seventy-two hours after an ip pump implantation, serum was obtained by a terminal retroorbital bleed from anesthetized mice. Immediately after euthanasia, bilateral thyroid lobes were excised and preserved in 0.35 mL lysis buffer (RLT; QIAGEN N.V.) in −70°C until RNA isolation.

In the second series of experiments, we administered L-T3 (5 μg/d, once daily, 10–12 AM; Sigma-Aldrich) by ip injection for 4 days and, with a 1-day lag, we delivered vehicle (formulation number 7) or ANTAG3 for 3 days (2 mg/d) by an ip-implanted osmotic pump. On the morning of the fourth day, we gave an acute ip injection of ANTAG3 (2 mg) or vehicle 4 hours before administering a single ip injection of M22 (0.5 μg) or PBS. We obtained serum and excised the thyroid glands 24 hours after the injection of M22.

Data were analyzed using GraphPad Prism 5 for Windows (GraphPad Software). Statistical analyses were performed by a Student's *t* test or one-way ANOVA; $P < .05$ was considered significant. Data are expressed as mean ± SE.

Results

Figure 1 illustrates the structure of ANTAG3 and shows that ANTAG3 is a selective antagonist of TSH stimulation of cAMP production at the human TSHR. The IC_{50} of ANTAG3 for stimulation by an EC_{50} of bovine TSH was 2.1 μM (95% confidence interval (CI) of 1.3–3.3 μM). ANTAG3 inhibited TSH stimulation by 76% (95% confidence interval of 70%–95%). In contrast, ANTAG3 inhibition of LH and FSH stimulation was less than 15% for LH and less than 30% for FSH at 30 μM.

We began in vivo studies by determining whether ANTAG3 had any effect on FT4 levels in control mice. We administered ANTAG3 to naïve female BALB/c mice and were not able to measure a consistent effect, most likely because the variability in control levels was too large. We next established a model of continuous stimulation of endogenous TSH secretion by administering 2.4 μg TRH per day for 3 days ip by an osmotic pump (Figure 2A). TRH administration increased the FT4 from 2.64 ± 0.12 ng/dL to 3.48 ± 0.26 ng/dL ($P < .005$). Figure 2(B and C), illustrates that the levels of FT4 elevated by TRH were lowered by ANTAG3 administration by 44%, and the levels of TPO and NIS mRNAs by 75% and 83%, respectively ($P < .001$). TG, DIO2, and TSHR mRNA levels did not significantly change with ANTAG3 administration.

We next attempted to develop a model that is more relevant to GD while at the same time buffering the variability in the measurements. We decided to inhibit endogenous TSH effects by administering T_3 (15) and then to stimulate the thyroid glands of mice by administering the thyroid-stimulating monoclonal antibody M22 (16) (Figure 3A). In a series of preliminary experiments, T_3 administration decreased serum FT4 from 2.64 ± 0.12 ng/dL to 0.26 ± 0.016 ng/dL, and M22 increased FT4 3.6- ± 0.30-fold. In two repeat experiments (n = 10–15 mice per group) (Figure 3B), M22 increased serum FT4 level 2.8-fold and ANTAG3 lowered the FT4 level back to 1.7-fold of the control, a 38% inhibition. In the same animals, M22 increased the levels of mRNAs for NIS and TPO by 4.0- and 6.1-fold, respectively, and ANTAG3 lowered these levels by 73% and 40%, respectively (Figure 3C). TG, DIO2, and TSHR mRNA levels, on the other hand, were not changed by either stimulant or antagonist intervention.

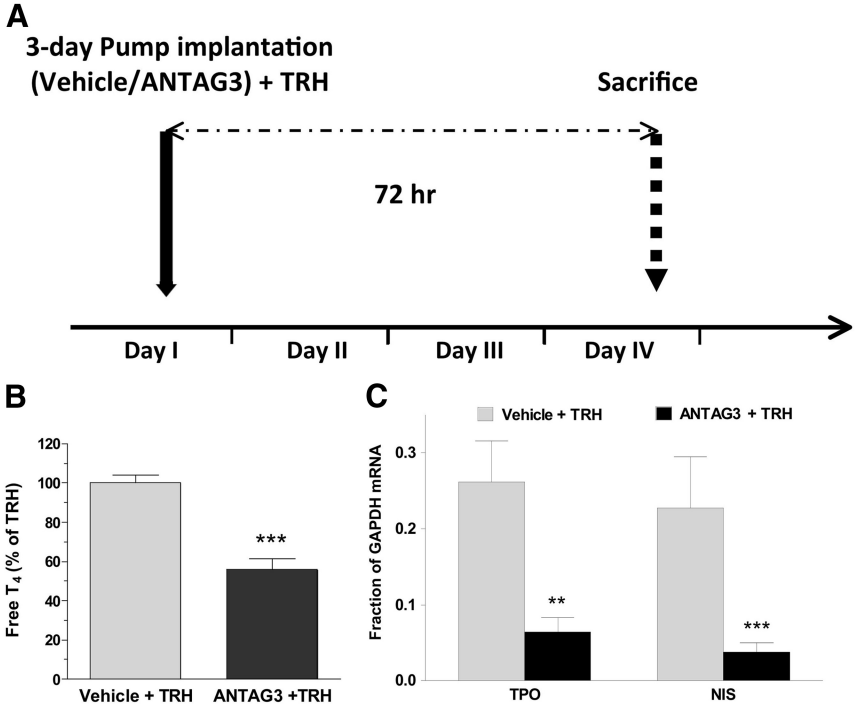

Figure 2. ANTAG3 lowers serum FT4 levels and thyroidal mRNAs for TPO and NIS in mice continuously stimulated by TRH. A, TRH (2.4 μg/d for 3 d) was administered ip via osmotic pump with or without ANTAG3 (2 mg/d). The animals were euthanized on day 4. B, Serum FT4 levels. C, mRNA levels of TPO and NIS in thyroid gland lysates. The bars represent the mean ± SE of TRH-treated (n = 20) and ANTAG3+TRH-treated mice (n = 16) in three experiments. ***, $P < .001$, **, $P < .01$ ANTAG3 vs vehicle.

Discussion

Herein we provide evidence that ANTAG3 is a selective TSHR antagonist that is effective at inhibiting TSHR-

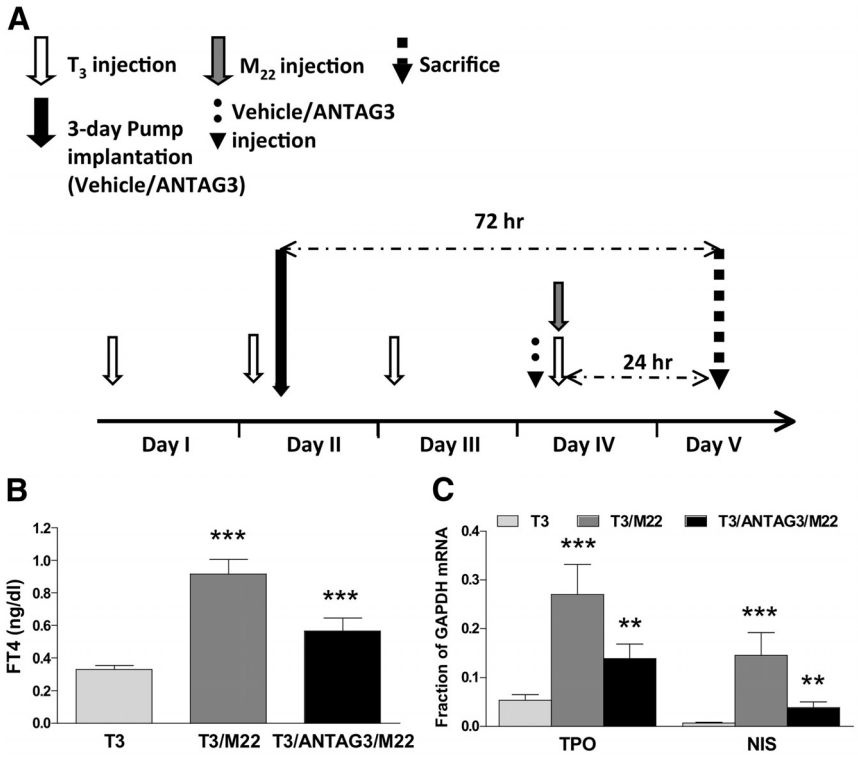

Figure 3. ANTAG3 lowers serum FT4 levels and thyroidal mRNAs for TPO and NIS in mice stimulated by a single injection of M22. A, T_3 (5 μg/d for 4 d) was administered by daily ip injection, and ANTAG3 was given for 3 days (2 mg/d) via an osmotic pump. On day 4, the animals were given an ip injection of vehicle or ANTAG3 (2 mg) and 4 hours later an ip injection of M22 (0.5 μg) or vehicle. The animals were euthanized 24 hours after the M22 injection on day 5. B, Serum FT4 levels. C, mRNA levels of TPO and NIS in thyroid gland lysates. The bars represent the mean ± SE of vehicle only (n = 10), vehicle + M22-treated (n = 15), and ANTAG3 + M22-treated mice (n = 14) in two experiments. ***, $P < .001$, **, $P < .01$ T_3 vs T_3/M22 and T_3/M22 vs T_3/ANTAG3/M22.

mediated responses in two mouse models. In a model cell culture system, ANTAG3 inhibited TSH-stimulated cAMP signaling with a half-maximal inhibitory concentration of 2.1 μM but had only a small effect on LH- and FSH-stimulated cAMP production at 30 μM. In an in vivo mouse model of thyroid gland stimulation by endogenous TSH, ANTAG3 inhibited the elevation in serum FT4 and the elevations of mRNAs for TPO and NIS caused by the continuous administration of TRH. In a mouse model of thyroid gland stimulation by a thyroid-stimulating monoclonal antibody, ANTAG3 inhibited the elevation in FT4 and the elevations of TPO and NIS mRNAs caused by M22. Although acute administration of M22 antibody is not a model for GD, these data indicate that ANTAG3 can inhibit thyroid activation by a stimulating antibody. A TSHR-binding monoclonal antibody was reported to lower total T_4 levels in mice (17). To our knowledge, this is the first demonstration in any animal model of a TSHR-selective, small-molecule antagonist that is effective in inhibiting thyroid gland stimulation. These findings make it likely that an antagonist like ANTAG3 would be able to inhibit TSHR signaling in vivo in other tissues that express TSHRs including orbital fibroblasts/preadipocytes and adipocytes, which appear to play a role in the pathogenesis of Graves' ophthalmopathy (18) and that orbital cell-specific genes may serve as biomarkers for antagonist efficacy. We suggest therefore that a TSHR antagonist like ANTAG3 could be used to treat Graves' hyperthyroidism and ophthalmopathy.

In conclusion, we have developed a selective TSHR antagonist that is effective at inhibiting TSHR responses in the thyroid gland in an animal model. Further development of both the TSHR antagonist and an animal model with an appropriate readout for Graves' ophthalmopathy are needed to translate small molecule, drug-like TSHR antagonists to clinical usage.

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