Effect of thyromimetic GC-1 selective signaling on reproductive and lactational performance in the hypothyroid rat

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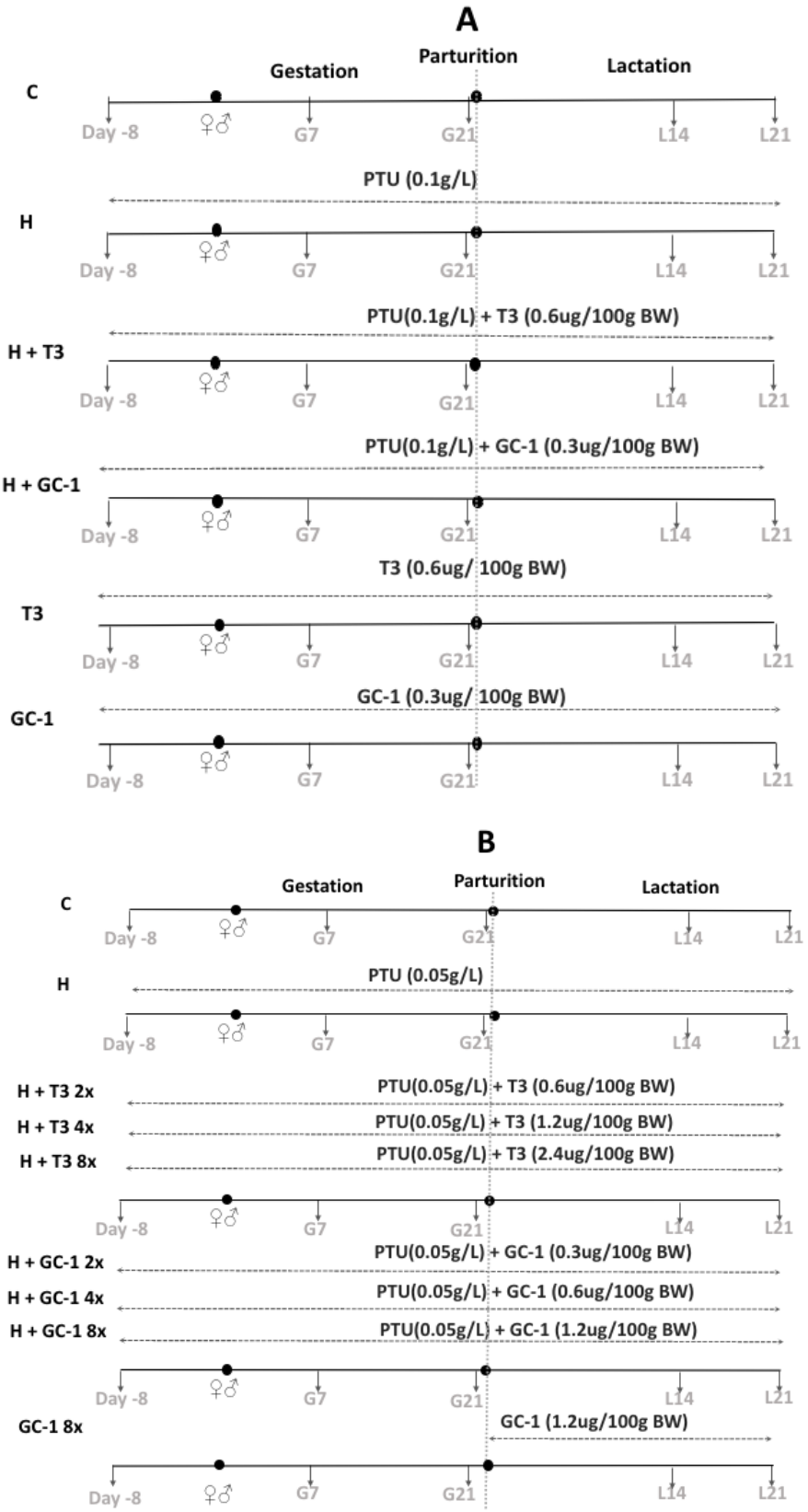
Supplemental information.
Reagents

GC-1 was kindly provided by Dr Thomas Scanlan, Oregon Health & Science University. 6-propyl-2-thiouracil (P3755) and 3,3′,5-Triiodo-L-thyronine sodium salt T3 (T6397) were purchased from Sigma-Aldrich.

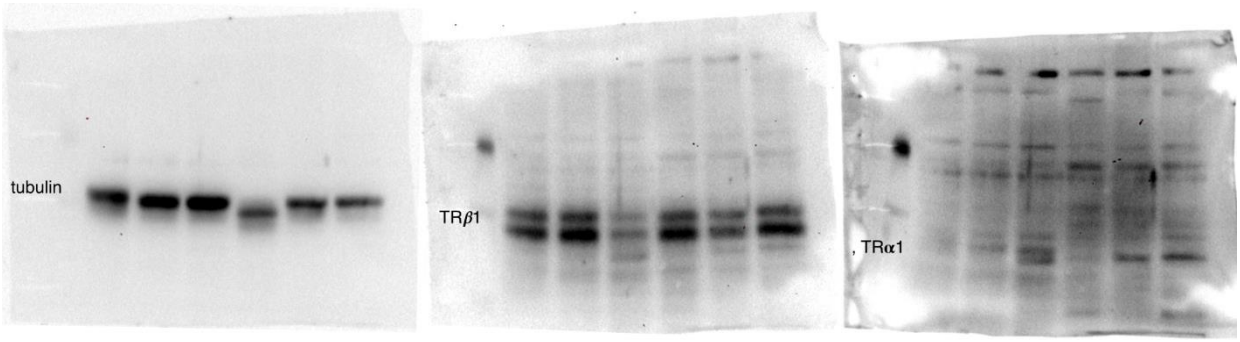
Supplemental Figure 1. Experimental design



Supplemental Figure 1: Experimental Design: animal groups, timeline and treatment (drugs and concentrations). Upper panel **A,** corresponds to the experimental design that evaluates: TSH and T3 serum concentrations during gestation and lactation in rats treated with thyromimetic GC-1 or T3 (Fig_1); effects of T3 or GC-1 on reproductive performance of hypothyroid and euthyroid female rats (Fig_2); thyroid hormone receptor protein expression in reproductive tissues (Fig_3); and the effect of maternal GC-1 and T3 administration on the regulation of thyroid axis, growth and survival of pups. Lower panel **B**, corresponds to the experimental design that evaluates the effect of maternal GC-1 and T3 administration on the regulation of thyroid axis, growth and survival of pups (Fig_5). ♀♂ day of mating with a fertile male. G7, G14 and G21 are days of gestation 7, 14 and 21, respectively. L14 and L21 are days of lactation 14 and 21, respectively.

Western blot analysis of TH receptors (TR)

TRα1 and TRβ1, the predominant TH binding receptors, were analyzed by Western blot in order to confirm their protein expression and to show their differential pattern of expression simultaneously in the same animal. Protein samples were isolated from CL, uterus, placenta and mammary gland of the same animal on day G19, homogenized in 10 volumes of lysis buffer containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.5% IGEPAL, 50mM sodium fluoride, and a protease inhibitor cocktail (Sigma P8340, Sigma-Aldrich, St. Louis, MO). The lysates were incubated on ice for 30 minutes and then for another 30 minutes on ice on a rocking platform. Lysates were centrifuged at 10,000 rpm for 15 minutes at 4ºC, and the supernatant was considered whole cell extract. Protein content was assessed by the bicinchoninic acid method (BCA; Pierce, Rockford, IL). The whole cell extract was aliquoted and stored at -80ºC. Before loading, the proteins were boiled for 10 minutes in sample buffer. Then, 50 µg of proteins were separated in a 7.5% (w/v) acrylamide gel by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked in 5% (w/v) nonfat milk in tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20. Blots were probed overnight in 2.5% bovine serum albumin (BSA) with the appropriate dilution of each of the primary antibodies [TRβ1 (J52; 1/1000) from Santa Cruz; TRα1 (PA1-211A; 1/1000) from Thermo Scientific (Waltham, MA), and a-tubulin (T6040; 1/12000) from Sigma-Aldrich]. The membranes were washed 3 times for 5 minutes in TBS-T and incubated with 1:5000 dilution of a peroxidase conjugated secondary antibody (anti-mouse horseradish peroxidase [HRP] from Cell Signaling [Beverly, MA] and anti-rabbit HRP from Santa Cruz Biotechnology [Dallas, TX]) for 1 hour at room temperature. The blots were washed, developed by chemiluminescence, and the images were captured using a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA).

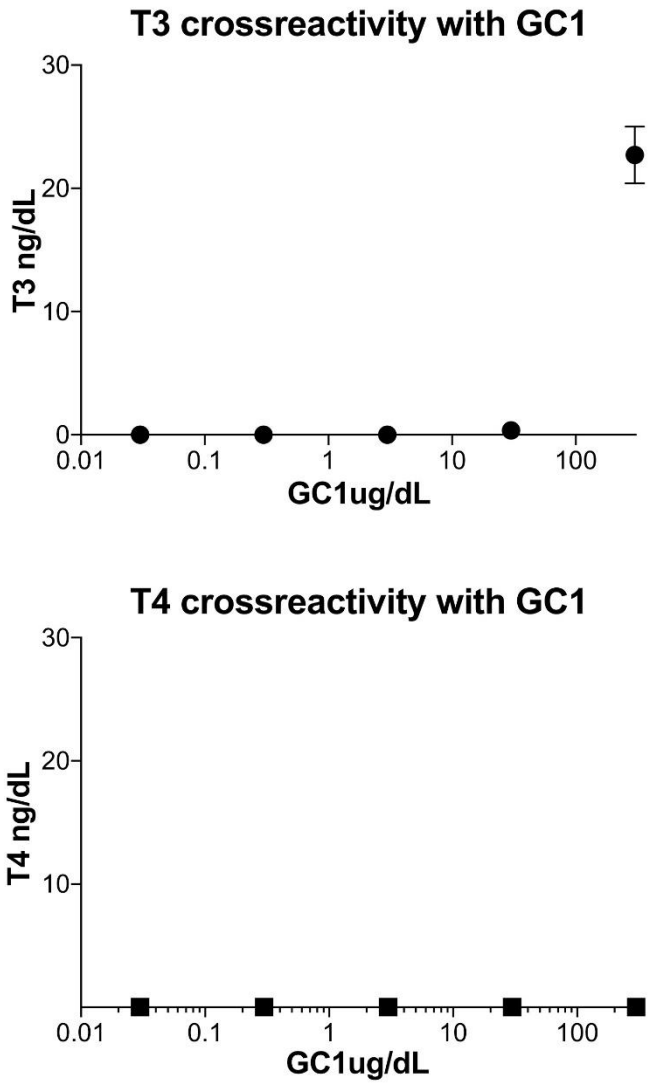


Supplemental Figure 2. Uncropped western blot images of Figure 3.

Hormone determinations

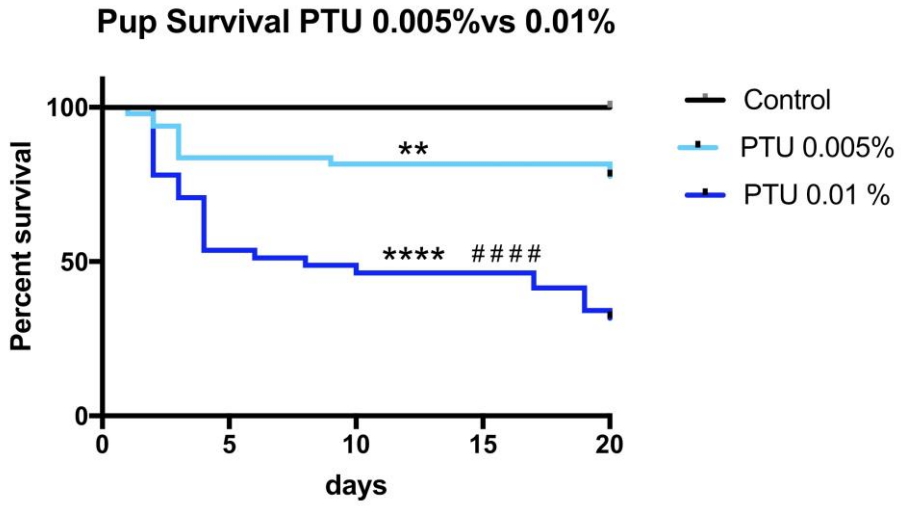
TSH concentration was measured by double antibody radioimmunoassay using materials generously provided by A. F. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA). The hormones were radio-iodinated using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat TSH RP-3 standard preparation, with a measurement range between 0.25-256 ng/mL serum. The inter- and intra-assay coefficients of variation were <10%.
T3 and T4 concentrations were measured by radioimmunoassay using commercial kits for total hormones (IM1579 and IM1447, respectively, from IMMUNOTECH (Beckman Coulter, Czech Republic)). Inter- and intra-assay coefficients of variation were <10%, with measurement ranges between 0.02-7.8 ng/mL for T3 and 8.25-310.8 ng/mL for T4.

Supplemental Figure 3



Supplemental Figure 3. Cross-reactivity of GC-1 with T3 and T4 on RIA determination. Determination of T3 and T4 in samples of charcoal-stripped serum from rats containing 0.03, 0.3, 3 or 300 ug/dL of GC-1. Each dilution was performed in triplicate. The GC-1 dosis administered daily to the rats does not cross-react with T3 or T4 determination by radioimmunoassay.

Supplemental Figure 4



Supplemental Figure 4. Effect of 6-propyl-2-thiouracil (PTU) maternal administration on rat pup survival. Kaplan-Meier survival curves of pups during lactation. Statistical differences between groups were evaluated using the log-rank (Mantel-Cox) test. Hypothyroidism in the mothers was induced by administering PTU in the drinking water at concentrations of 0.01 and 0.005 %. Control n= 42, PTU 0.005% n= 49 and PTU 0.01% n=41 pups per group. **p<0.01, ****p<0.0001 with respect to control group; ####p<0.0001 with respect to PTU 0.005% group.