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Semisynthesis and pharmacological activities of thyroxine analogs: Development of new angiogenesis modulators

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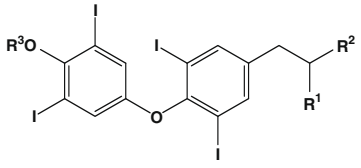
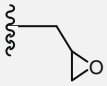
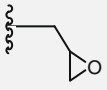
ABSTRACT

Novel thyroxine analogs with hindered phenol, amino and carboxylic acid groups have been synthesized and the effects of the synthesized compounds on angiogenesis using the chick chorioallantoic membrane and mouse matrigel models have been tested. Pharmacological profiles revealed that thyroxine tolerates numerous modifications on the amino group and remains active. These results provide the rationale for the selection of a novel thyroxine nanoparticle precursor.

Angiogenesis is as a vascular process that involves the induction of growth of new blood vessels through cell adhesion to the extra cellular matrix (ECM).[1] For the treatment of aneurysm, heart attack (e.g., acute myocardial infarction), stroke, or peripheral artery disease, there has been a critical need for an orally active compound to boost angiogenesis with an optimal therapeutic index and minimal impact on other physiological processes.[2] In the chick chorioallantoic membrane (CAM) assay, the thyroid hormone T_4 (2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propionate, thyroxine) and the T_4 analogs T_3 (2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propionate), and GC-1 ([4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylphenoxy]acetic acid) stimulate angiogenesis as effectively as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF). T_4 (Table 1, compound **1**) and T_4 analogs have also been identified as potent activators of integrin $\alpha_v\beta_3$, a cell adhesion molecule.[3–5] The relative pK_a values (dissociation constants) of the functional groups of T_4 have been estimated to be approximately 9.0 for the NH_2 (amino) group and 2.0 for the COOH (carboxylic acid) group. The PhOH (phenol) group is a weakly basic site as determined by fluorescent quenching ($pK_a = 6.2$).[6] The goal of the current study was to semisynthesize a new water-soluble formulation of thyroxine that could be used in conjunction with targeted nanoparticle drug delivery to stimulate angiogenesis. This novel nanoformulation

Table 1
Structures of T_4 and T_4 analogs **7–15**



Compounds	R^1	R^2	R^3
Thyroxine 1	$-NH_3^{\oplus}$	$-COO^{\ominus}$	–H
7	–NHBoc	–COOH	–H
8	–NHBu	–COOH	–H
9	–NHAc	–COOH	–Ac
10	–NHMe	–COOH	–H
11	–NHEt	–COOH	–H
12	–NHEt	–COOEt	–H
13	–NHBoc	–COOTiPS	–H
14	–NHBoc		
15	–NHBoc	–COOTiPS	

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would be unable to gain access to the cell, and thus work only at the cell surface. As part of this approach, suitable intermediates were synthesized to determine the relative importance of the NH_2 group and the distance between the phenyl and COOH group of T_4 to pro-angiogenic activity.

A nanoscale particle consisting of poly-L-lactide-co-glycolide (PLGA) and polyvinyl alcohol (PVA) saturated on its surface with NH_2 groups was selected as the nanoformulation for T_4.[7] *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC)-assisted amide formation between the lyophilized polymer and ethylenediamine was found to be the best pathway for obtaining NH_2 groups on the surface of the nanoparticle. Association of the activated hormone with the nanoparticle would be mediated through an epoxide-based linker.[8] Thus, T_4 was activated by alkylation after the formation of carbon- or silicon-based esters and protection of the NH_2 group. The compounds were then tested for their ability to stimulate angiogenesis. It is possible that the epoxide, when linked to the OH (hydroxyl) of the PhOH, interacted with a serine or tyrosine residue at or near the binding site of the integrin receptor.[9,10]

A computer-aided chain ramification design in which the propionic amino acid end of T_4 had been modified was used to determine the fit of derivatives into the integrin binding site.[11] Several candidate modified analogs were identified that would be amenable to a linear synthesis strategy of nanoparticle precursor (Table 1).[12–14] The program was allowed to insert the designed T_4 analogs into

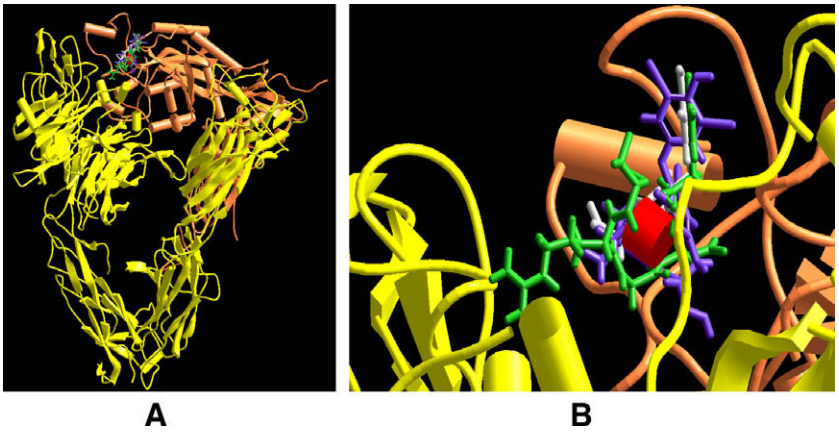

Figure 1. (A) Ribbon diagram of the two protein subunits (yellow and orange) of integrin $\alpha_v\beta_3$. (B) Docking of the natural ligand (green), tetrac (white), and compound **7** (purple) between the two protein subunits.

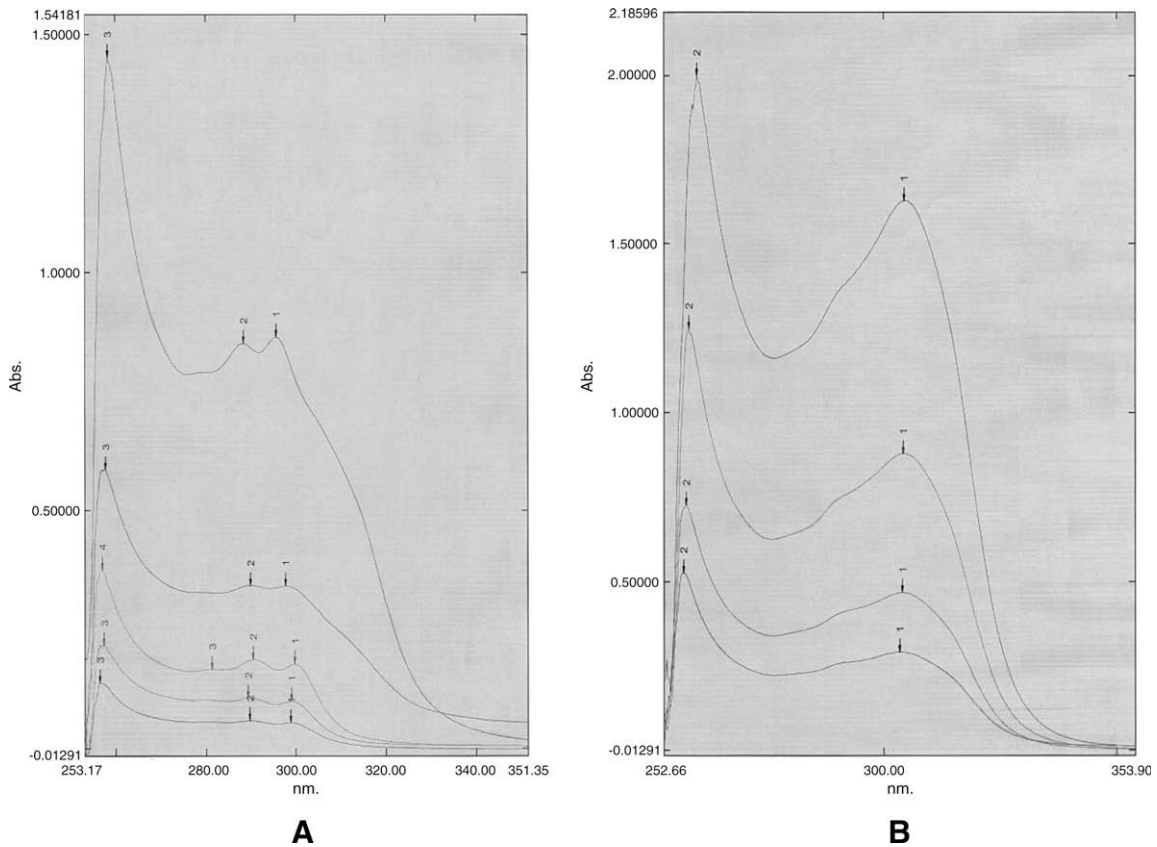

Figure 2. (A) UV-vis spectra of compounds **12**, **13**, **14**, **15** and **9** (from top to bottom). (B) UV-vis spectra of compounds **7**, **11**, **8** and **10** (from top to bottom).

Table 2
Pro-angiogenic effects of T_4 analogs in the CAM model

Treatment	Mean branch points[a]	Mean % of stimulation[b] (comparing to PBS)
PBS (control)	73 ± 6	–
FGF (1.25 μg/mL)	130 ± 11[c]	78 ± 7[c]
T_4	137 ± 8[c]	88 ± 7[c]
8	98 ± 11[d]	34 ± 8[d]
9	122 ± 4[c]	67 ± 5[c]
10	105 ± 7[d]	44 ± 6[d]
11	136 ± 13[c]	86 ± 9[c]
FGF + 12	46 ± 2[e]	−38 ± 4[c] (inhibition)
13	76 ± 7	4 ± 6
14	114 ± 11[c]	56 ± 7[c]
15	98 ± 8[d]	34 ± 7[d]

[a] Data represent the means ± SEM (n = 8).
[b] All thyroid analogs were tested at a concentration of 10 μg/CAM.
[c] P <0.01 as compared to PBS (control).
[d] P <0.05.
[e] P <0.01 as compared to FGF.

the integrin binding pocket with no constraints imposed for the site of docking. In other words, the 'ligands' were not assigned a particular place to bind. Fifty conformers of the lowest energy were considered for each compound probed. For example, of the fifty conformers for compound **7**, 38 of the 'ligands' positioned themselves at the same place (Fig. 1). A choice of different anhydrides [Boc_2O (di-*tert*-butyl dicarbonate), Bu_2O (butyric anhydride), Ac_2O (acetic anhydride)], silylating agents [TMSCl (trimethylsilyl chloride), TiPSCl (triisopropylsilyl chloride)], and electrophilic alkylating agents [MeI (iodomethane), EtI (ethyl iodide), epibromohydrin] has been shown to modulate the functional groups according to nucleophilicity and steric hindrance.

For the synthesis of novel analogs, a comparative study of yield and kinetics of acylation and alkylation of T_4 was carried out using three different methods (A, B and C; Supplementary data) in order to find the most suitable and reproducible synthesis protocol. In method A, TEA (triethylamine) (1.0 equiv) was used to activate the NH_2 group prior to the addition of functional agents. In method B, the organic base was replaced by an inorganic base (Cs_2CO_3; cesium carbonate) (1.0 equiv). In method C, no bases were used and the reactions were allowed to proceed until thermodynamic equilibrium was obtained. Cs_2CO_3 slightly increased the rate of the reactions, but not as much as TEA (method A). The best yields were obtained when T_4 zwitterion was solely used (Method C), particularly for compound **7**.

The COOH group was then modified into a carbon- or silicon-based ester.[15] Interestingly, the carboxylate group of analog **7** reacted better with a cluttered silicon-based esterifying agent. It reacted quantitatively with the TiPSCl group, but did not react with the more electrophilic TMSCl group.[16] Although recent data suggested the existence of a putative siliconium ion, no further studies were done to elucidate the mechanism of this reaction.[17,18] T_4 was protected using the Boc reagent and then esterified with TiPSCl (1.0 equiv) in situ after another round of deprotonation using TEA (1.0 equiv) in anhydrous THF (tetrahydrofuran) at room temperature to yield compound **13** (Supplementary data, Fig. S2). This compound was isolated, epibromohydrin was added in excess, and then anhydrous reflux conditions were applied to generate compound **15**, which was subsequently isolated after purification by silica gel chromatography (40% overall yield). Interestingly, while the general trends in the ultraviolet spectra of all pure products were very similar, certain features were different. Monosubstituted analogs of the COOH group had one characteristic absorption wavelength of 304 nm in addition to one near 250 nm, whereas any disubstituted analog had two maxima in the region at 290 and 299 nm (Fig. 2).

To investigate the structure–activity relationships of the derivatives, the effect of each compound on angiogenesis was compared to T_4 using two model systems: the CAM model, a widely used in vivo model in which the chicken embryonic membrane is exposed to putative stimulatory and inhibitory compounds;[15,19] and the matrigel assay, in which putative angiogenesis-inducing and blocking compounds are incorporated into cold liquid Matrigel, which is then injected subcutaneously, serving as a scaffold for the formation of new blood vessels.[16,20]

Compounds were considered strong agonists if they induced the same level of stimulation as the reference molecules (Table 2). T_4 analogs substituted at the NH_2 and OH ends had a stimulatory ef-

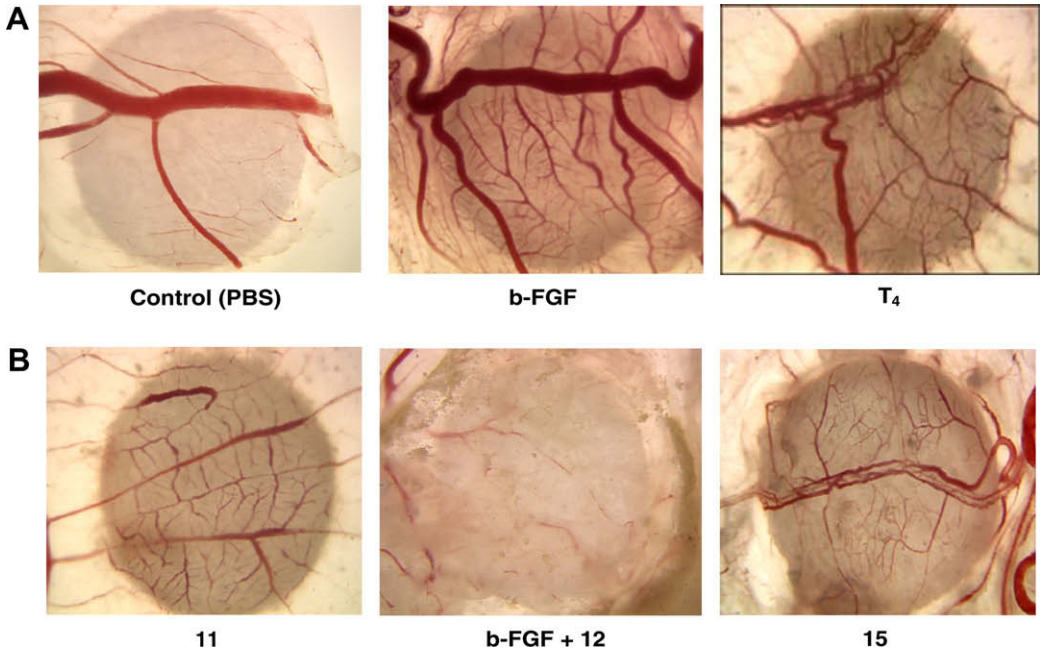

Figure 3. Representative images showing the effect on angiogenesis of b-FGF, T_4 and **11** (pro-angiogenic); **12** (anti-angiogenic); and **15** (no activity).

fect on new blood vessel formation, but the effect was not comparable to the reference compounds. The extent of angiogenesis induced by compounds in which the NH_2 group was protected with small moieties such as Me (methyl) (**10**), or Et (ethyl) (**11**) was similar (105 and 136 branch points, respectively) to that of free T_4 (137 branch points) and FGF (130 branch points) (Fig. 3 and Table 2). Similar activity was observed for analogs **8** and **9**. Conversely, disubstituted compounds with highly lipophilic groups (**12**, **13**, and **15**) were less effective than the control. In fact, compound **12** inhibited the effect of FGF. Compound **14**, in which T_4 was disubstituted with a hydrogen bond acceptor at the COOH end, stimulated angiogenesis to a lesser extent than T_4. Synthesized analogs **8**, **9**, **10**, **11**, **12**, **13**, **14**, and **15** were also tested for their ability to stimulate angiogenesis, as measured by the production of hemoglobin (Hb), in the matrigel assay. T_4 significantly induced angiogenesis (Table 3). T_4 analogs **8**, **9**, and **10** also induced angiogenesis at levels comparable to T_4 (62, 95, and 87%, respectively). T_4 analog **11** induced angiogenesis to a lesser extent (45%). There was no apparent stimulation of angiogenesis by analogs **12**, **13**, **14**, and **15**. T_4 analog **12** appeared to inhibit T_4-induced angiogenesis (Table 4).

The results of the CAM and matrigel assays (Tables 2–4) indicated that the greater the extent of hindrance of the NH_2 group, the greater the inhibition of T_4 pro-angiogenic activity. That is, all mono-acylated and mono-alkylated analogs demonstrated clearly diminished stimulation. A second substitution on the PhOH end (**9**) did not significantly reduce the potency of the drug, which suggested that this part of the molecule did not participate in binding. A second substitution at the COOH end, on the other hand, prevented stimulation completely. One analog, compound **12**, had the surprising effect of entirely inhibiting FGF and T_4 activity. Compared to analog **11**, the presence of an Et group at the acid end of **12** appeared to disable the molecule in terms of stimulating the production of new blood vessels and antagonized the effects of T_4 to the same extent as tetrac ([4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]acetic acid) (Table 4).

In summary, synthetic modifications of thyroid hormone led to a significantly improved understanding of the functional importance of the COOH and NH_2 groups of the molecule for pro-angiogenic activity. A useful compound has been validated as a precursor for a water-soluble nanoparticle formulation of the hormone. The results suggested the OH of the PhOH group as the best site for condensation with a nanoparticle.

Table 3
Pro-angiogenic effects of T_4 analogs on angiogenesis in the mouse matrigel model

	Hemoglobin (mg/mL) ± SEM[a]	Mean stimulation of angiogenesis[b] (%)
Control	0.3 ± 0.1	—
T_4	0.9 ± 0.2	100 ± 22
8	0.7 ± 0.2	62 ± 38
9	0.9 ± 0.2	95 ± 33
10	0.8 ± 0.2	87 ± 33
11	0.6 ± 0.2	45 ± 33
13	0.3 ± 0.1	0 ± 17
14	0.3 ± 0.1	0 ± 17
15	0.3 ± 0.1	0 ± 17

[a] Data represents the means ± SEM (n = 6–7).
[b] All thyroid analogs were tested at a concentration of 10 μg/matrigel plug.

Table 4
Anti-angiogenic effects of T_4 analog **12** on angiogenesis in the mouse matrigel model

	Hemoglobin (mg/mL) ± SEM[a]	Mean inhibition of angiogenesis[b] (%)
Control	0.3 ± 0.1	—
T_4	1.2 ± 0.4	—
T_4 + Tetrac	0.4 ± 0.1	89 ± 11
T_4 + **12**	0.4 ± 0.1	89 ± 11

[a] Data represents the means ± SEM (n = 25).
[b] T_4, **12**, and tetrac were tested at a concentration of 10 μg/matrigel plug.

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Supplementary data

Supplementary data (synthesis and analytical characterization of all compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.011.

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11. Docking of **7** and tetrac to $\alpha_v\beta_3$ was performed using Autodock version 2.4. This program allows flexibility in the ligand structure but uses a rigid body protein approximation in order to speed up the calculation. The 1L5G.pdb file for the protein used for docking was taken from the Brookhaven database. Partial charges for protein atoms were taken from the AutoDock version of the AMBER force field5. For the model ligands, flexibility was allowed for all torsion angles. Partial atomic charges required for the docking calculation were obtained by ab initio quantum chemistry calculations using the HyperChem® program (HyperCube). Grids of probe atom interaction energies were computed first; 84 Å side grids with a spacing of 0.7 Å were used for all the modules. The ligand probes were then docked by simulated annealing. Typically, the 50 lowest energy coordinate sets were extracted for each ligand type and used for visualization in the software WebLab Viewer (Molecular Simulation, Inc).
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19. To assess angiogenesis in the CAM model system, 10 day-old fertilized chicken eggs (Sunrise Farms, Inc, Catskill, NY) were incubated at 37 °C in 55% relative humidity. In the dark, with the help of a candling lamp, a small hole was punctured in the area of the shell covering the air sac using a hypodermic needle. A second hole was punctured on the wider side of the egg above an avascular area of the embryonic membrane. An artificial air sac was created below the second hole by gently applying a vacuum to the first hole using a small rubber squeeze bulb. An approximately 1.0 cm^2 window was cut in the shell over the dropped CAM with the use of a mini drill. Filter disks (filter paper #1, Whatman International, Ltd, United Kingdom) were soaked in 3 mg/mL cortisone acetate solution (in 95% ethanol) and then air-dried under sterile conditions. Sterile filter disks were saturated with L-T_4 or FGF; control disks

were saturated with PBS without Ca and Mg. Using sterile forceps, one filter was placed on the CAM and then the window was sealed with transparent tape. After 1 hr, 10 μL of inhibitor were applied topically to the L-T_4 or FGF-stimulated CAM. After 48 h, CAM tissue directly beneath the filter disk was harvested and placed in a 35 mm petri dish. CAM sections were examined using an SV6 stereomicroscope (Carl Zeiss MicroImaging, Inc) at 50× magnification. Digital images were captured using a 3-CCD color video camera system (Toshiba America, Inc, New York, NY). The images were analyzed using Image-Pro Plus (Media Cybernetics, Inc). The number of blood vessel branch points within a circular region superimposed on the area of the filter disk was counted for each section (10 eggs per condition).

20. *Mouse matrigel model of angiogenesis*: Normal male mice (C57BL/6NCr) 6–8 weeks of age weighing approximately 20 g were purchased from Taconic Farms, Inc. Animals were housed four per cage under controlled environmental conditions (20–24 °C, 60–70% humidity and a 12 h light/dark cycle) and provided with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the VA Hospital of Albany, NY, USA. Mice were allowed to acclimate for 5 days prior to the start of treatment. Matrigel (BD Biosciences, San Jose, CA) was thawed overnight at 40 °C and placed on ice. Aliquots of matrigel were placed into cold polypropylene tubes to prevent the matrigel from solidifying, and compound was added to the matrigel with or without an agonist. Matrigel plugs were subcutaneously injected at three sites in each animal (total of 100 μL/animal). At day 14 after plug implant, all animals were sacrificed in a CO_2 chamber, and matrigel plugs were collected. Plug hemoglobin content was analyzed from three implants per mouse ($n = 6$ per group) to measure angiogenesis. For hemoglobin measurement, matrigel plugs were carefully stripped of any remaining peritoneum. The plugs were placed into a 0.5 mL tube of ddH2O and homogenized for 5–10 min. The samples were subjected to centrifugation at 4000 rpm for 10 min, and then 50 μL of the supernatant were mixed with 50 μL of Drabkin's reagent and allowed to sit at room temperature for 15–30 min, at which point 100 μL of the mixture were placed in a 96-well plate. Absorbance was measured with a Microplate Manager ELISA reader at 540 nm. Hemoglobin concentration was determined by comparison with a standard curve in mg/mL.