Bioorganic & Medicinal Chemistry Letters xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of MR-49, a deiodinated analog of tetraiodothyroacetic acid (tetrac), as a novel pro-angiogenesis modulator

Mehdi Rajabi [a], Thangirala Sudha [a], Noureldien H. E. Darwish [a,b], Paul J. Davis [a,c], Shaker A. Mousa [a,*]

[a] The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, 1 Discovery Drive, Rensselaer, NY, USA
[b] Faculty of Medicine, Mansoura University, Mansoura, Egypt
[c] Department of Medicine, Albany Medical College, Albany, NY, USA

ARTICLE INFO

Article history:
Received 20 April 2016
Revised 23 June 2016
Accepted 24 June 2016
Available online xxxx

Keywords:
L-Thyroxine (L-T_4)
3,3′,5-Triiodo-L-thyronine (L-T_3)
Tetraiodothyroacetic acid (tetrac)
Deiodination
Pro-angiogenesis

ABSTRACT

The tyrosine-based hormones 3,3′,5-triiodo-L-thyronine (L-T_3) and L-thyroxine (L-T_4) that are produced by the thyroid gland control metabolic functions. Iodothyronine deiodinase enzymes convert L-T_4 to L-T_3, the form of thyroid hormone critical to genomic actions within cells and regulation of metabolism, and to reverse-L-T_3, a hormone isoform that is largely inactive. We used tertiary amines in a study of deiodination based on derivatives of tetraiodothyroacetic acid (tetrac)—a naturally occurring derivative of L-T_4—to mimic the action of the iodothyronine deiodinases and deiodination of the outer ring iodines. Deiodinated tetrac, MR-49, was found to be pro-angiogenic, with this activity exceeding that of L-T_3 and L-T_4 in a hemoglobin Matrigel® plug assay of angiogenesis. Tetrac is anti-angiogenic via several nongenomic pathways, and the present studies of MR-49 reveal the critical contribution of outer ring iodines to the angiogenic properties of thyroid hormone analogues, which may have utility as pro-angiogenic pharmaceuticals.

The tyrosine-based hormone 3,3′,5-triiodo-L-thyronine (L-T_3) is secreted by the thyroid gland or derived in the periphery from L-thyroxine (L-T_4) (Fig. 1) via outer ring deiodination. It controls a large number of physiological processes such as normal differentiation, growth, and integration of metabolic functions in most cells.[1–8] L-T_4 is thus a prohormone for L-T_3 in terms of intracellular hormonal actions, but at a receptor on the cell surface of blood vessel cells, L-T_4 acts as hormone and is pro-angiogenic.[9,10] Deiodination of the inner ring of L-T_4 may also occur, yielding 3,3′,5-triiodo-L-thyronine (reverse-L-T_3, also called rL-T_3), a generally inactive form of the hormone. Types 1 and 2 iodothyronine deiodinases (D1 and D2) catalyze the conversion of L-T_4 to L-T_3 (Fig. 2A). Both L-T_3 and rL-T_3 may then undergo further monodeiodination (outer ring 5′-iodine of rL-T_3, inner ring 5-iodine of L-T_3) to 3,3′-T_2, and these transformations are due either to type 3 iodothyronine deiodinase (D3) (L-T_3) or D1 (rL-T_3).[3,11–13]

Angiogenesis is the process of formation and growth of new blood vessels through relevant cell division and cell–cell adhesion and adhesion of cells to the extracellular matrix (ECM) proteins. In endothelial and vascular smooth muscle cells, angiogenesis is dependent upon plasma membrane integrin expression. Results of studies of thyroid hormone (L-T_4, L-T_3) and hormone analogues such as GC-1 ([4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylphenoxy]acetic acid) in the chick chorioallantoic membrane (CAM) model of angiogenesis led to the description of a cell surface receptor for thyroid hormone on integrin $\alpha v\beta_3$ by which the hormone can stimulate angiogenesis as effectively as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF)[9,10] (Fig. 2B).

It was also shown that the pro-angiogenic thyroid hormone signal at plasma membrane integrin $\alpha v\beta_3$ was transduced into downstream intracellular actions by mitogen-activated protein kinase, specifically, extracellular-signal-regulated kinase 1/2 (ERK1/2).[1] We have described the pro-angiogenesis activity of thyroid hormone analogues such as L-T_4, L-T_3, diiodothyropropionic acid (DITPA) and GC-1, a nuclear thyroid hormone receptor-β (TRβ)-selective agonist, in the human dermal microvascular endothelial cell (HDMEC) tubule and CAM models.[9,10,14–17] We also extensively studied angiogenesis activity of tetraiodothyroacetic

Abbreviations: CAM, chick chorioallantoic membrane; FGF, fibroblast growth factor; D1 and D2, types 1 and 2 iodothyronine deiodinases; ECM, extracellular matrix; ERK1/2, extracellular-signal-regulated kinase 1/2; L-T4, L-thyroxine; L-T3, 3,3′,5-triiodo-L-thyronine; rL-T3, reverse-L-T_3; tetrac, tetraiodothyroacetic acid.

* Corresponding author. Tel.: +1 518 694 7397; fax: +1 518 694 7567.
E-mail address: shaker.mousa@acphs.edu (S.A. Mousa).

http://dx.doi.org/10.1016/j.bmcl.2016.06.064
0960-894X/

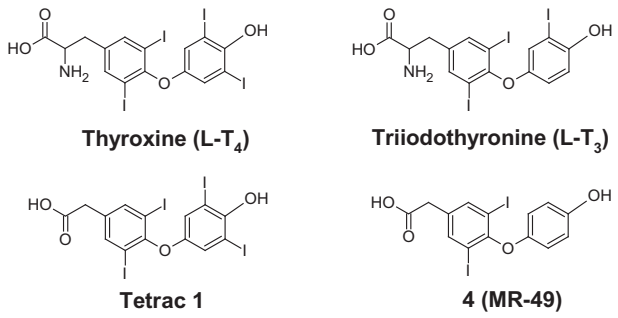


Figure 1. Chemical structures of L-thyroxine (L-T_4), triiodothyronine (L-T_3), tetrac (tetraiodothyroacetic acid), and MR-49 (deiodinated tetrac).

acid (tetrac), a deaminated derivative of T_4 (Figs. 1 and 2B) that, acting alone, is anti-angiogenic at the receptor site and that also blocks pro-angiogenic activity of thyroid hormone analogues, such as those listed above.[15,18–21] As shown in Figure 2B—in the absence of L-T_4 and L-T_3—tetrac surprisingly inhibited the vascular budding in response to VEGF and b-FGF in chick and human endothelial cell assays. Thus, we found that its anti-angiogenic activity is apparently related to its binding to the cell surface receptor for thyroid hormone on integrin $\alpha v\beta_3$. The receptor is proximal to the Arg-Gly-Asp (RGD) recognition site of the integrin, a site that via crosstalk is critical to the binding of vascular growth factors by their specific protein receptors[10,15] that are adjacent to the integrin. Cellular uptake of tetrac is prevented by covalent conjugation of its outer ring hydroxyl to 120–150 nm nanoparticles. This tetrac-nanoparticulate acts exclusively at the hormone receptor on the integrin and is anti-angiogenic, confirming the role of this receptor in the results mentioned above.[10,22,23] Tetrac also has been studied for its tumor cell-targeting properties for drug delivery. For example, Lee et al.[24] synthesized tetrac-linked pegylated liposomes that displayed heightened accumulation in integrin $\alpha v\beta_3$-expressing melanoma cells and increased cell uptake of a chemotherapeutic agent (edelfosine) compared to non-targeted liposomes. In tumor xenografted animals, administration of tetrac-linked liposomes decreased tumor growth and increased animal survival compared to control (non-targeted) lipsomes. The same group[25] synthesized ^{64}Cu-labeled tetrac-conjugated liposomes for PET imaging of tumor angiogenesis. They found tetrac/^{64}Cu-DOTA-liposomes had higher uptake by human aortic endothelial cells in vitro and clear in vivo visualization of tumors in micro PET images, compared to ^{64}Cu-DOTA-liposomes.

In the current study, we synthesized a new analog of tetrac that expresses pro-angiogenic rather than anti-angiogenic activity. To mimic the action of the three iodothyronine deiodinases, deiodination of tetrac has been explored with the use of tertiary amines. **MR-49** (Fig. 1) is the result of this approach to deiodination of

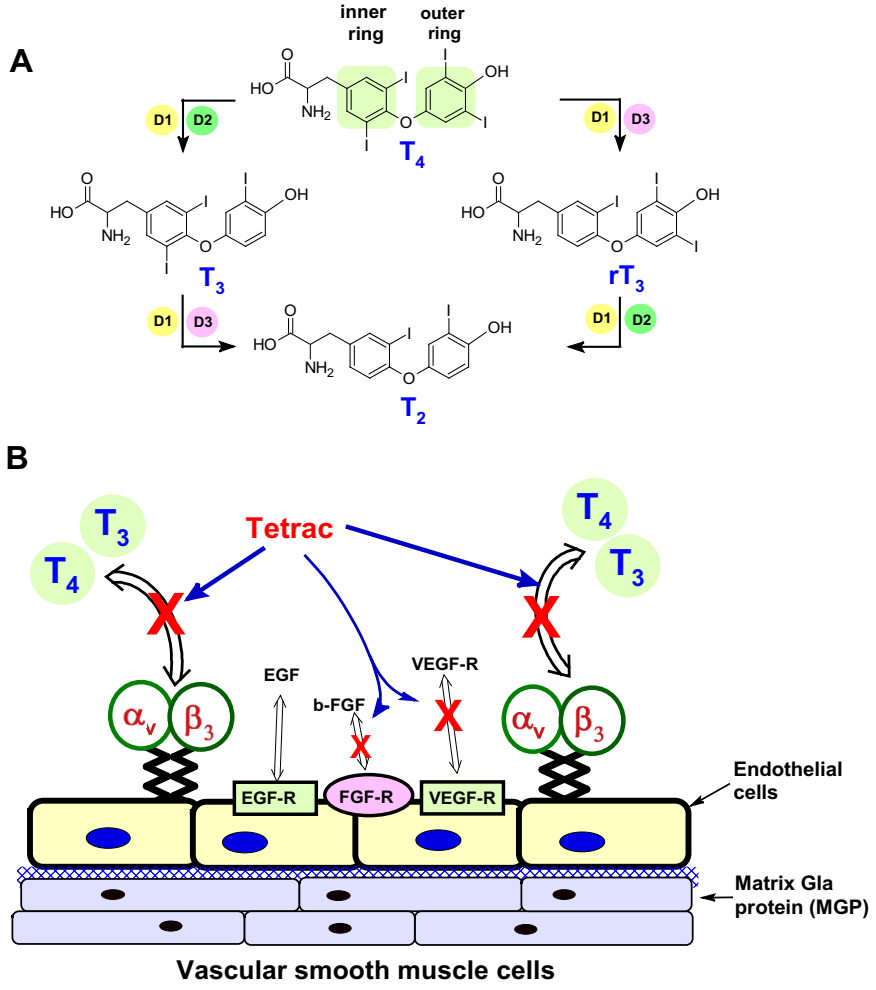


Figure 2. (A) The metabolism of L-T_3 and L-T_4 into active and inactive intermediates involves the action of 3 types of deiodinases. (B) Pro-angiogenic actions of thyroid hormone (L-T_4 and L-T_3) on endothelial cells and vascular smooth cells that are initiated at the cell surface receptor for the hormone on the extracellular domain of integrin $\alpha v\beta_3$. A thyroid hormone analogue that is inhibitory at the integrin receptor, tetraiodothyroacetic acid (tetrac), is anti-angiogenic.

Please cite this article in press as: Rajabi, M.; et al. *Bioorg. Med. Chem. Lett.* (2016), http://dx.doi.org/10.1016/j.bmcl.2016.06.064

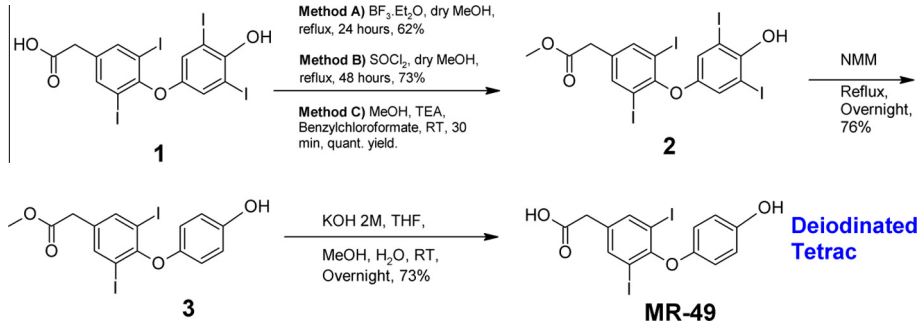
tetrac, and its angiogenic activity has been found to exceed that of L-T_3 and L-T_4 in standard angiogenesis assays.

We began by protecting the phenylacetic acid end of tetrac **1**, employing three different methods. In method A, using a protecting method published by Desage-El Murr et al.,[26] a solution of tetrac **1** and boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$) in methanol was stirred in dry conditions at ambient temperature for 24 h. The reaction was quenched by adding saturated $NaHCO_3$ solution and compound **2** (methyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate) was obtained in 62% yield. In method B,[27] protection of tetrac **1** was achieved with thionyl chloride ($SOCl_2$) in anhydrous methanol under 2 days reflux; compound **2** was precipitated by adding water and then was collected by vacuum filtration, followed by recrystallization as a lipophilic ester in 62% yield. We also tried another method (C) for synthesis of ester tetrac **2**, suspending the tetrac in anhydrous methanol and adding triethylamine (TEA), followed by adding benzylchloroformate and stirring for 30 min. Compound **2** was obtained with a 73% yield by filtering, removing the solvent, and recrystallization. Fourier transform infrared spectroscopy (FTIR) and ^1H and ^{13}C NMR spectroscopy were used to confirm the synthesized compounds. The carbonyl (C=O) of the acid group of tetrac, which had an FTIR peak at 1693 cm^{-1}, shifted to 1719 cm^{-1} after protection of acid to ester. ^1H NMR data showed a 3-proton peak at 3.74 ppm and ^{13}CNMR data showed a peak at 52.1 ppm, which is assigned to the methyl group of ester tetrac **2** (Scheme 1).

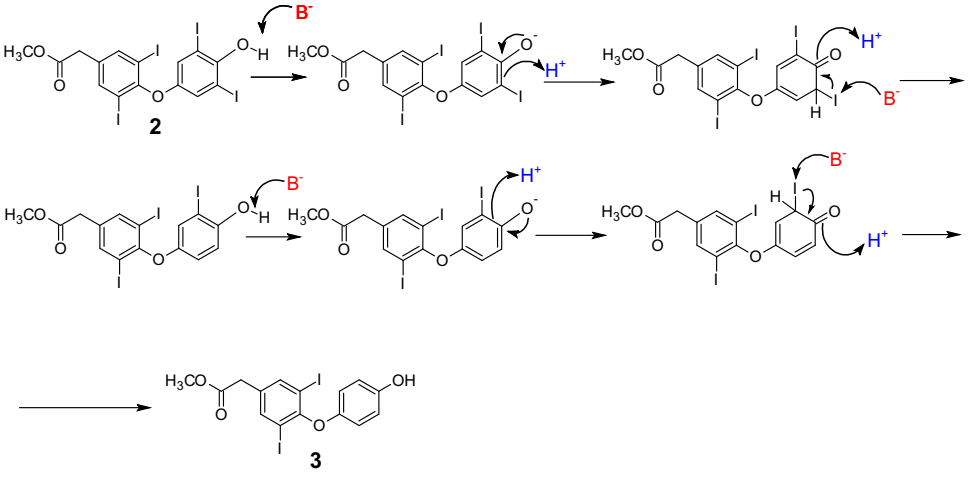
Our efforts continued with dehalogenation of compound **2** by selectively removing the iodines from the outer ring. Dehalogenation of aromatic halides and, particularly hydrodehalogenation, refers to efficient replacement of a halogen with hydrogen and that has wide applications in many of the same areas as basic hydrogenation. Similar to debromination, different reducing agents such as metal hydrides,[28] catalytic hydrogenation, and acidic conditions such as Zn/HCl, Pd/C, Zn or $SnCl_2/CH_3CO_2H$, and $CuCN/FeCl_3$ have been used for deiodination of aromatic halides.[29] Several studies have reported deiodination by using derivatives of 2,4-diiodophenol to mimic the action of the iodothyronine deiodinases.[30,31] Consequently, following the work of Talekar et al.,[29] our plan was to deiodinate compound **2** via initial deprotonation of the phenolic OH group by tertiary amines like *N*-methylmorpholine (NMM), and then the resulting anion abstracts a proton to form tautomeric dienone. Subsequently, nucleophilic attack on the iodine atom by either hydroxide ion or base results in the deiodinated product (Scheme 2). We also tried using other tertiary bases like TEA, *N*-methyl-2-pyrrolidone (NMP), and pyridine, but NMM showed higher yield of deiodination than other bases.

Results from ^1H NMR showed that tertiary base catalyzed removal of two iodines from the *ortho* position of the phenol ring of **2** (outer-ring) without any effect on iodines of the inner ring. After hydrodehalogenation, a new peak appeared at 6.67 ppm, which is assigned to hydrogen at the *ortho* position and was associated with disappearance of the hydrogen peak assigned to the *meta* position at 7.12 ppm that shifted to 6.76 ppm (Fig. 3).

Finally, the compound **MR-49** was obtained by deprotection of compound **3** using KOH in THF/methanol (1:1). Organic solvent was evaporated completely and the mixture was neutralized by 1 M HCl. The precipitate was then collected by vacuum filtration and dried to give a white powder of [4-(4-hydroxyphenoxy)-3,5-diiodophenyl]acetic acid **MR-49** in 73% yield.



Scheme 1. Synthesis of deiodinated tetrac (MR-49) from tetraiodothyroacetic acid (tetrac).



Scheme 2. Possible mechanism for deiodination of protected tetrac **1**, using base.

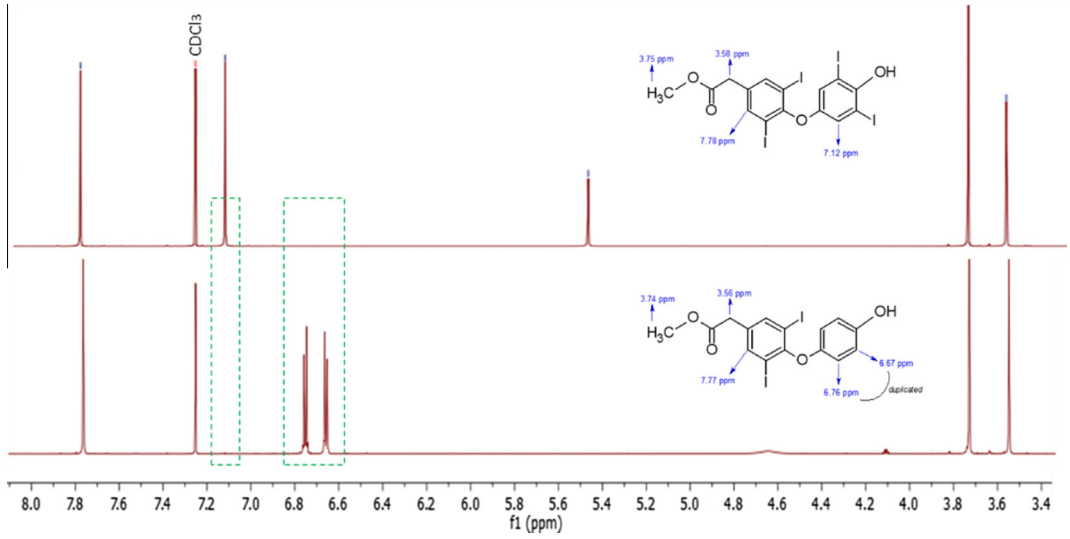


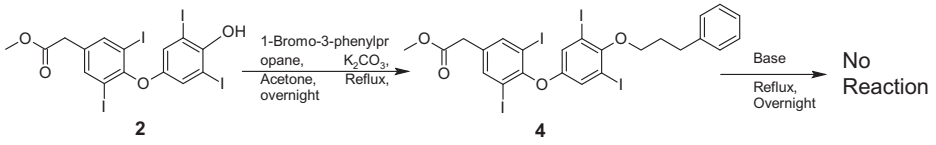
Figure 3. NMR analysis of deiodination of protected tetrac **2**.

To confirm that our deiodination method has an effect only on the outer-ring of **2** and also to study the role of the hydroxyl group in deiodination, we protected the hydroxyl group of ester tetrac **2** with 1-bromo-3-phenylpropane to synthesize the compound **4** (methyl {4-[3,5-diiodo-4-(3-phenylpropoxy)phenoxy]-3,5-diiodophenyl}acetate) in the presence of potassium carbonate and acetone with 76% yield. Compound **4** was subsequently used in reaction with tertiary bases, but we didn't observe any conversion. This confirmed that the presence of the phenolic OH group is key for initial deprotonation of the phenolic OH group by tertiary amines to cause the anion to form a tautomeric dienone, which undergoes nucleophilic attack (Scheme 3).

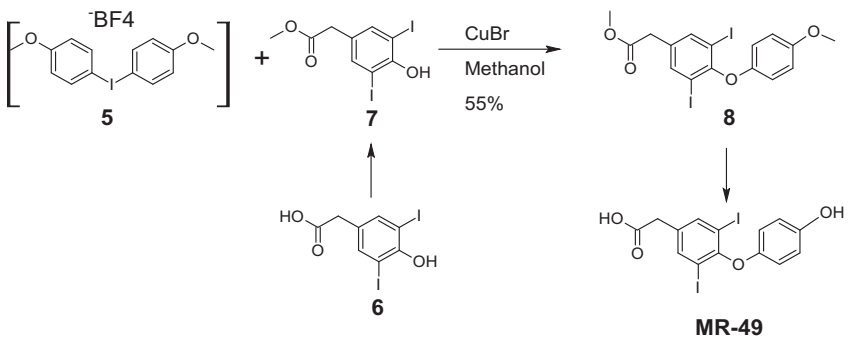
We also tried using an alternative method for synthesis of **MR-49**, similar to the published method of Ziegler and Marr,[32] but instead of using dianisyliodonium bromide, we started from bis (4-methoxyphenyl)iodonium tetrafluoroborate **5** in reaction with [4-hydroxy-3,5-diiodophenyl]acetic acid **7**, which was obtained from (4-hydroxy-3,5-diiodophenyl)acetic acid **6** according to the procedure in method B and also confirmed by Balyon, et al. (see Supplementary information). Compound **5** was reacted with **7** in the presence of trimethylamine, copper bromide, and absolute methanol stirred overnight at room temperature. Copper was removed through a short aluminum hydroxide column, followed by washing with benzene, DCM, and methanol. The collected solvent was removed, and the resulting crude methyl [3,5-diiodo-4-(4-methoxyphenoxy)phenyl]acetate **8** was used without any purification and boiled in acetic acid and hydriodic acid for 3 h for deprotection, resulting in **MR-49** (Scheme 4).

To assess the potency of the pro-angiogenic activity of MR-49 versus tetrac and T_3, their activities were evaluated in a Matrigel® plug and a hemoglobin assay. The Matrigel® plug assay is an efficient method to evaluate either stimulators or inhibitors of angiogenesis in vivo.[33] Matrigel® is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains growth factors and extracellular matrix components.[34]

We evaluated the effect of **MR-49** in vivo using a mouse model (BALB/c mice). Male BALB/c mice aged 5–6 weeks weighing between 18 and 20 g were purchased from Envigo (Indianapolis,



Scheme 3. Synthesis of methyl {4-[3,5-diiodo-4-(3-phenylpropoxy)phenoxy]-3,5-diiodophenyl}acetate **4** and reaction with tertiary base.



Scheme 4. Alternative method for synthesis of MR-49.

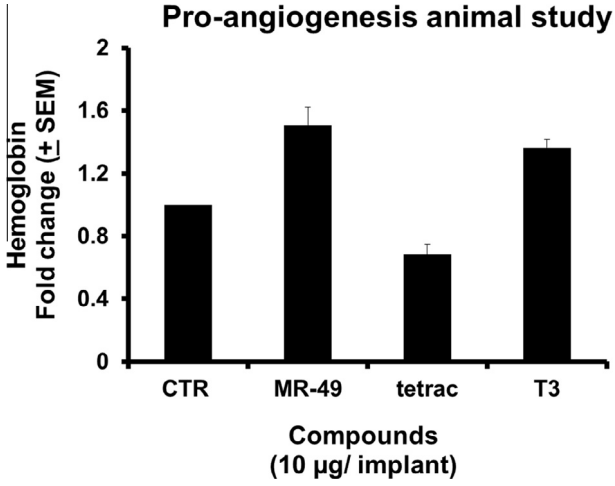


Figure 4. Matrigel® plug assay of angiogenesis (pro-angiogenesis effect of MR-49, L-T_3, and tetrac). Values are expressed as mean ± SEM versus control vehicle (CTR). Values are expressed as mean ± SEM. Tetrac significantly inhibited hemoglobin content in the Matrigel plug (index of angiogenesis) while MR-49 or L-T3 increased it.

IN). All animal studies were conducted at the animal facility of the Albany College of Pharmacy and Health Sciences, Albany, NY in accordance with and approved by institutional guidelines for humane animal treatment and according to the current guidelines. Mice were maintained under specific pathogen-free conditions and housed under controlled conditions of temperature (20–24 °C), humidity (60–70%), and 12 h light/dark cycle with ad libitum access to water and food. Mice were allowed to acclimatize for 5 d prior to the start of study.

Mice were dived into 4 groups: control, MR-49, L-T_3, and tetrac. Pro-angiogenic (**MR-49** and L-T_3) and anti-angiogenic substances (tetrac) were added to Matrigel® and injected subcutaneously in the mice (Matrigel® plug assay), and the control group received vehicle. There were 4 implants per mouse with a concentration of 10 µg/implant and 2 mice per group. After 7–10 days, the Matrigel plug was removed and new blood vessel formation in the Matrigel® could be visualized. Vessel formation was quantified by measuring the hemoglobin concentration (Drabkin method[35]). Drabkin's solution consists of potassium ferricyanide, potassium cyanide, and sodium bicarbonate. In this method, hemoglobin is oxidized to methemoglobin by potassium ferricyanide, which in turn reacts with potassium cyanide to form cyanmethemoglobin.[36] Spectrophotometric readings were taken at 540 nm in a spectrophotometer. Hemoglobin values were calculated from a hemoglobin curve prepared using a hemoglobin standard.

We found that MR-49 and L-T_3 were associated with increased hemoglobin concentration, consistent with new blood vessels' formation. MR-49 increased hemoglobin concentration by 1.6-fold in comparison to the control group, while L-T_3 increased hemoglobin concentration to a lesser extent (1.4-fold). On the other hand, tetrac was associated with decreased hemoglobin concentration by about 50% (Fig. 4).

In conclusion, we used tertiary amines in a study of deiodination based on derivatives of tetrac—a naturally occurring derivative of T_4—to mimic the action of the iodothyronine deiodinases and deiodination of the *ortho*-iodohydroxylated arenes of thyroid hormone analogs. Iodothyronine deiodinase enzymes convert T_4 to L-T_3, the form of thyroid hormone critical to genomic actions within cells and to regulation of metabolism, and to reverse L-T_3, a hormone isoform that is largely inactive. T_4, itself, initiates a number of nongenomic actions of thyroid hormone at the cell surface. Deiodination was obtained via initial deprotonation of the phenolic OH group by tertiary amines and consequent anion extraction of a proton to form a tautomeric dienone. Subsequent nucleophilic attack on the iodine atom by either hydroxide ion or base resulted in a deiodinated product of tetrac, designated MR-49. Tetrac is anti-angiogenic via several nongenomic pathways and the present studies of MR-49 reveal the critical contribution of outer ring iodines to the angiogenic properties of thyroid hormone analogues.

Funding sources: Partial funding was from NanoPharmaceuticals, LLC.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.064. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Brent, G. A. *J. Clin. Invest.* **2012**, *122*, 3035.
2. Cheng, S. Y.; Leonard, J. L.; Davis, P. J. *Endocr. Rev.* **2010**, *31*, 139.
3. Gereben, B.; Zavacki, A. M.; Ribich, S.; Kim, B. W.; Huang, S. A.; Simonides, W. S.; Zeold, A.; Bianco, A. C. *Endocr. Rev.* **2008**, *29*, 898.
4. Hollenberg, A. N.; Forrest, D. *Cell Metab.* **2008**, *8*, 10.
5. Horn, S.; Heuer, H. *Mol. Cell Endocrinol.* **2010**, *315*, 19.
6. López, M.; Alvarez, C. V.; Nogueiras, R.; Diéguez, C. *Trends Mol. Med.* **2013**, *19*, 418.
7. Warner, A.; Mittag, J. *J. Mol. Endocrinol.* **2012**, *49*, R29.
8. van der Deure, W. M.; Peeters, R. P.; Visser, T. J. *J. Mol. Endocrinol.* **2010**, *44*, 1.
9. Mousa, S. A.; Davis, F. B.; Mohamed, S.; Davis, P. J.; Feng, X. *Int. Angiol.* **2006**, *25*, 407.
10. Luidens, M. K.; Mousa, S. A.; Davis, F. B.; Lin, H. Y.; Davis, P. J. *Vasc. Pharmacol.* **2010**, *52*, 142.
11. Martínez-Sánchez, N.; Alvarez, C. V.; Fernø, J.; Nogueiras, R. C. D.; López, M. *Best Prac. Res. Clin. Endocrinol. Metab.* **2014**, *28*, 703.
12. Dentice, M.; Marsili, A.; Zavacki, A.; Larsen, P. R.; Salvatore, D. *Biochim. Biophys. Acta* **2013**, *1830*, 3937.
13. Drigo, R. A. E.; Fonseca, T. L.; Werneck-de-Castro, J. P. S.; Bianco, A. C. *Biochim. Biophys. Acta* **2013**, *1830*, 3956.
14. Davis, P. J.; Davis, F. B.; Mousa, S. A. *Curr. Cardiol. Rev.* **2009**, *5*, 12.
15. Mousa, S. A.; Bergh, J. J.; Dier, E.; Rebbaa, A.; O'Connor, L. J.; Yalcin, M.; Aljada, A.; Dyskin, E.; Davis, F. B.; Lin, H. Y.; Davis, P. J. *Angiogenesis* **2008**, *11*, 183.
16. Mousa, S. A.; O'Connor, L.; Davis, F. B.; Davis, P. J. *Endocrinology* **2006**, *147*, 1602.
17. Mousa, S. A.; O'Connor, L. J.; Bergh, J. J.; Davis, F. B.; Scanlan, T. S.; Davis, P. J. *J. Cardiovas. Pharmacol.* **2005**, *46*, 356.
18. Glinskii, A. B.; Glinsky, G. V.; Lin, H. Y.; Tang, H. Y.; Sun, M.; Davis, F. B.; Luidens, M. K.; Mousa, S. A.; Hercbergs, A. H.; Davis, P. J. *Cell Cycle* **2009**, *8*, 3562.
19. Yoshida, T.; Gong, J.; Xu, Z.; Wei, Y.; Duh, E. J. *Exp. Eye Res.* **2012**, *94*, 41.
20. Yalcin, M.; Bharali, D. J.; Dyskin, E.; Dier, E.; Lansing, L.; Mousa, S. S.; Davis, F. B.; Davis, P. J.; Mousa, S. A. *Thyroid* **2010**, *20*, 281.
21. Yalcin, M.; Dyskin, E.; Lansing, L.; Bharali, D. J.; Mousa, S. S.; Bridoux, A.; Hercbergs, A. H.; Lin, H. Y.; Davis, F. B.; Glinsky, G. V.; Glinskii, A.; Ma, J.; Davis, P. J.; Mousa, S. A. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 1972.
22. Rajabi, M.; Srinivasan, M.; Mousa, S. A. Nanobiomaterials in Drug Delivery In *Nanobiomaterials in Drug Delivery: Applications of Nanobiomaterials*; Grumezescu, A., Ed.; Elsevier: Amsterdam, 2016; Vol. 9, p 1.
23. Srinivasan, M.; Rajabi, M.; Mousa, S. A. Nanobiomaterials in Cancer Therapy In *Nanobiomaterials in Cancer Therapy: Applications of Nanobiomaterials*; Grumezescu, A., Ed.; Elsevier: Amsterdam, 2016; Vol. 7, p 57.
24. Lee, S.; Kim, J.; Shim, G.; Kim, S.; Han, S. E.; Kim, K.; Kwon, I. C.; Choi, Y.; Kim, Y. B.; Kim, C. W.; Oh, Y. K. *J. Control. Rel.* **2012**, *164*, 213.
25. Kang, C. M.; Koo, H. J.; Lee, S.; Lee, K. C.; Oh, Y. K.; Choe, Y. S. *Nucl. Med. Biol.* **2013**, *40*, 1018.
26. Desage-El Murr, M.; Nowaczyk, S.; Le Gall, T.; Mioskowski, C. *Eur. J. Org. Chem.* **2006**, 1489.
27. Bridoux, A.; Cui, H. D.; Dyskin, E.; Yalcin, M.; Mousa, S. A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3259.
28. Czaplik, W. M.; Grupe, S.; Mayer, M.; Jacobi von Wangelin, A. *Chem. Commun.* **2010**, 6350.
29. Talekar, R. S.; Chen, G. S.; Lai, S. Y.; Chern, J. W. *J. Org. Chem.* **2005**, *70*, 8590.
30. Beck, C.; Jensen, S. B.; Reglinski, J. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1353.
31. Vasil'ev, A. A.; Engman, L. *J. Org. Chem.* **1998**, *63*, 3911.
32. Ziegler, H.; Marr, C. *J. Org. Chem.* **1962**, *27*, 3335.
33. Malinda, K. M. *Methods Mol. Biol.* **2009**, *467*, 287.
34. Lee, K. H.; Choi, H. R.; Kim, C. H. *J. Ethnopharmacol.* **2005**, *97*, 509.
35. Nuti, E.; Cantelmo, A. R.; Gallo, C.; Bruno, A.; Bassani, B.; Camodeca, C.; Tuccinardi, T.; Vera, L.; Orlandini, E.; Nencetti, S.; Stura, E. A.; Martinelli, A.; Dive, V.; Albini, A.; Rossello, A. *J. Med. Chem.* **2015**, *58*, 7224.
36. Balasubramaniam, P.; Malathi, A. *J. Postgrad. Med.* **1992**, *38*, 8.