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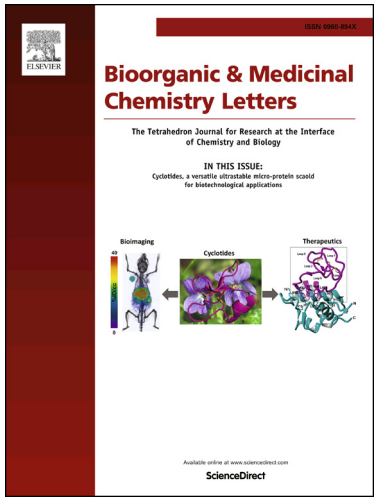

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Synthesis of new analogs of tetraiodothyroacetic acid (tetrac) as novel angiogenesis inhibitors for treatment of cancer

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ABSTRACT

In the angiogenesis process, integrins, which are members of a family of cell surface transmembrane receptors, play a critical role particularly in blood vessel formation and the local release of vascular growth factors. Thyroid hormones such as L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) promote angiogenesis and tumor cell proliferation via integrin αvβ3 receptor. At or near an arginine-glycine-aspartate (RGD) recognition site on the binding pocket of integrin $\alpha_v\beta_3$, tetraiodothyroacetic acid (tetrac, a deaminated derivative of T_4) is a thyrointegrin receptor antagonist and blocks the actions of T_3 and T_4 as well as different growth factors-mediated angiogenesis. In this study, we synthesized novel tetrac analogs by modifying the phenolic moiety of tetrac and tested them for their anti-angiogenesis activity using a Matrigel plug model for angiogenesis in mice. Pharmacological activity results showed that tetrac can accommodate numerous modifications and maintain its anti-angiogenesis activity.

KEYWORDS:

Angiogenesis, thyroid hormones, tetraiodothyroacetic acid, tetrac, integrin

Tumor angiogenesis is the formation of new blood vessel growth from the existing vasculature by cell adhesion to the extra cellular matrix (ECM), which results in tumor progression.[1,2] Rapid tumor cell proliferation produces environmental stresses such as a hypoxic, glucose-deprived environment that begins the angiogenic switch whereby tumor cells produce angiogenic activators including angiogenin, transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor, basic fibroblast growth factor (bFGF, FGF2), platelet-derived endothelial growth factor, and hepatocyte growth factor.[3]

Integrins are important transmembrane receptors that play a critical role in the angiogenesis process, particularly in blood formation and local release of vascular growth factors. They are members of a family of cell surface receptors that are immunoglobulin superfamily molecules or ECM proteins,[4] and consist of α and β chain units. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are the main receptor types involved in the angiogenesis process, especially in binding with angiogenesis modulators containing an arginine-glycine-aspartate (RGD) recognition site that binds to the integrin receptor.[5] There are several natural RGD-containing proteins such as fibronectin, fibrinogen, λ-receptor on E.coli, sindbis coat protein, and α-lytic protease protein[6] as well as cyclic RGD tripeptide (c-RGD) and c-RGD peptidomimetics[7] that show high binding affinity to the integrin recognition site.

Many studies have reported the effects of thyroid hormone analogs like L-thyroxine (T_4), which is a prohormone antecedent to 3,5,3'-triiodo-L-thyronine (T_3), on thyroid hormone receptor (TR) via binding to integrin $\alpha_v\beta_3$ and the effects of the angiogenesis activity of T_4 and T_3 with an interaction site that is located at or near the RGD recognition site of integrins (**Figure 1A**). Tetraiodothyroacetic acid (tetrac), a deaminated derivative of T_4, inhibited the pro-angiogenesis response of thyroid hormone by inhibiting the cell surface-initiated actions of T_4 and T_3 in the chick chorioallantoic membrane (CAM) and many other angiogenesis models.[8,9] Anti-proliferative activity of tetrac against other cancer cell lines such as human non-small cell lung cancer[10] have been investigated both *in vitro* and in xenografts and results showed anti-angiogenic activity at the integrin $\alpha_v\beta_3$ receptor-binding site. For example, Yoshida *et al.* found tetrac to be an effective inhibitor of retinal angiogenesis and of the pro-angiogenic effect of both erythropoietin (EPO) and VEGF on retinal endothelial cells; this suggested that tetrac (and

antagonism of integrin $\alpha_v\beta_3$) is a viable therapeutic strategy for proliferative diabetic retinopathy.[11]

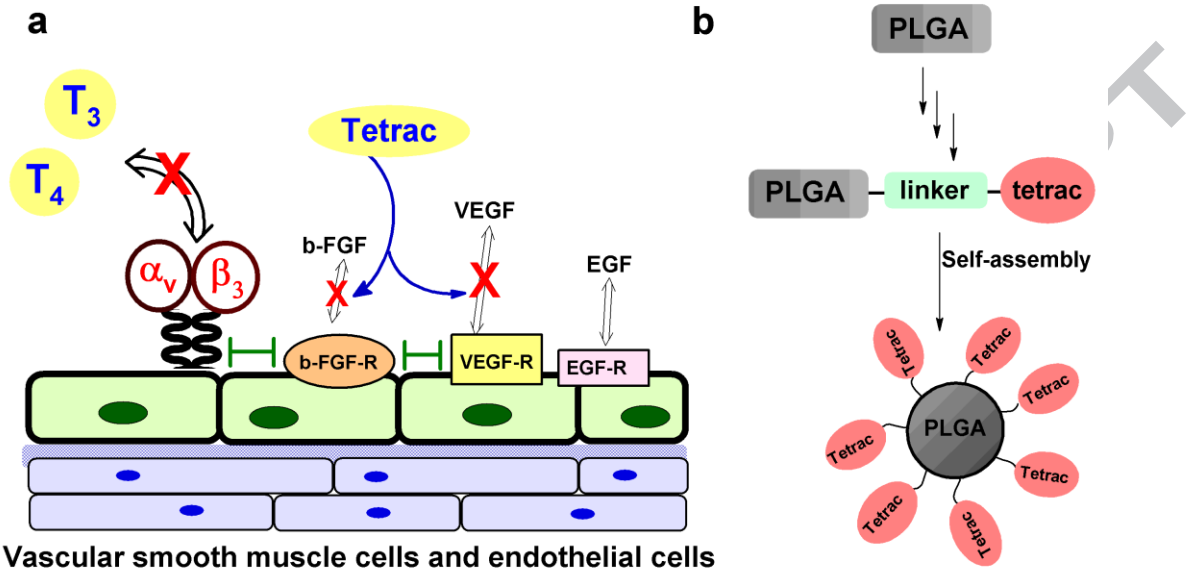


Figure 1. a) Pro-angiogenic activity of thyroid hormones T_4 and T_3 on vascular smooth and endothelial cells is initiated at the cell surface receptor (integrin) for the hormone on the extracellular domain of integrin $\alpha_v\beta_3$. Tetrac, a thyroid hormone analog, is inhibitory at the $\alpha_v\beta_3$ integrin receptor and is anti-angiogenic. **b)** Conjugation of tetrac to PLGA nanoparticle results in tetrac-nanoparticle that has shown anti-angiogenic activity.

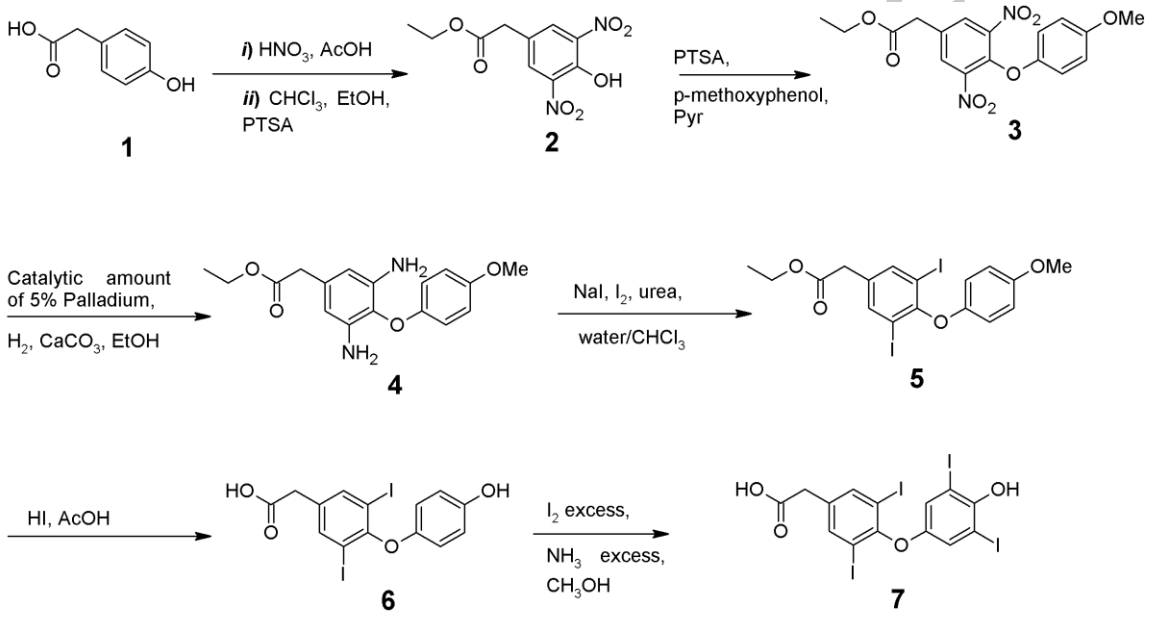
In order to limit tetrac to the cell surface thyroid hormone receptor and to provide optimized exposure of the biphenyl structure and acetic acid side chain of its inner ring to the receptor site on αvβ3, our group has conjugated tetrac to the polymeric nanoparticle (NP) poly (lactic-co-glycolic acid) (PLGA) via covalent binding (an amide bond formation) between tetrac's outer ring hydroxyl to NHS-modified PLGA, resulting in tetrac NP (Nano-diamino-tetrac; NDAT; Nanotetrac). This NP showed anti-angiogenic activity, confirming the role of the integrin receptor in the results explained above (**Figure 1B**).[8] Results from both *in vitro* and *in vivo* experiments for the treatment of drug-resistant breast cancer showed that NDAT is not able to enter into the cell nucleus but will enhance inhibition of tumor proliferation at a low-dose equivalent of free tetrac.[12] It has been concluded that NDAT has high potential as an anticancer agent,[13-15] with possible applications in the treatment of drug-resistant cancer. NDAT also inhibits the PI3-K and MAPK pathways[16,17] and blocks the expression of a panel of genes critical to cancer cell survival pathways and the epidermal growth factor receptor (*EGF-R)* gene.[18,19] We also designed and synthesized a novel derivative of tetrac that showed pro-angiogenic activity

rather than anti-angiogenic activity by mimicking the action of the iodothyronine deiodinases (the enzymes that convert T_4 to T_3). In this regard, the phenolic OH group of tetrac was initially deprotonated, and subsequent anion extraction of a proton formed a tautomeric dienone. Finally, nucleophilic attack on the iodine atom resulted in a deiodinated product of tetrac, designated MR-49.[20]

Knowing that the angiogenesis activity of tetrac is stimulated by FGF or VEGF without influencing the pre-existing blood vessels, we synthesized new tetrac analogs by modifying the phenolic moiety of tetrac and studied the structure-activity relationship. The phenolic hydroxyl group (-OH) of thyroid analogs is an important site for their modification and is a target site for converting tetrac to an integrin antagonist without any changes to the carboxylic acid moiety of tetrac. We previously synthesized diamino-tetrac, which showed anticancer/anti-angiogenic activity and was able to target the thyroid hormone-tetrac receptor on the extracellular domain of integrin $\alpha_v\beta_3$. Wu *et al.*[21] showed that conjugation of the phenolic hydroxy group (4'-OH) of T_4 with sulfate or glucuronic acid yielded the corresponding sulfated (T_4S) and glucuronidated (T_4G) hormone[22]; sulfate and glucuronic acid conjugations are useful to enhance the water solubility of many hydrophobic drugs and to enhance their excretion through urine and/or bile.[23] Similar to T_4S and T_4G, tetrac also undergoes sulfonation and glucuronidation, thus suggesting that the body can metabolize tetrac.[22]

As shown in **Scheme 1**, a large scale of compound **7**, tetrac, was synthesized according to the method of Wikinson[24] and optimized by our group.[25] The scheme starts with nitration of *p*-hydroxyphenylacetic acid **1** in the presence of nitric acid and acetic acid at 45°C to yield the first intermediate, dinitrophenylacetic acid, in 81% yield (m.p. 175-176°C). This was then converted to its ethyl ester **2** in 73% yield (m.p. 70-71°C) by dissolving in dry chloroform containing ethanol and *p*-toluene sulfonic acid under reflux conditions. Ethyl 3,5-dinitro-4-(4'-methoxyphenoxy)phenyl acetate **2** was mixed with *p*-toluene sulfonyl chloride in anhydrous pyridine and heated at 95-100°C for 30 min, and then *p*-methoxyphenol (temperature raised to 125°C for 2.5 h under nitrogen) was added to give the diphenyl ether derivative **3** in 63% yield. The two nitro groups of **3** were reduced and converted to amine **4** using a catalytic amount of 5% palladium, H_2, and calcium carbonate in ethanol as solvent. Compound **4** was then reacted with sodium iodide to convert the amine groups to iodine, resulting in compound **5** in 69 % yield, followed by hydrolysis and deprotection under reflux conditions using hydro-iodic acid (HI) as a

strong cleaving agent for facile removal of both the ester and O-methyl groups to form **6**. Acetic acid was used to avoid decomposition of HI into iodine by sulfuric acid, thereby avoiding the generation of unwanted side products in the reaction medium. Finally, tetrac **7** was obtained from the di-iodo compound **6** using iodine in methanolic-NH_3 with near quantitative yields. Regioselective iodination of the ring in compound **6** was facilitated by the transformation of the methyl ether group of compound **5** into phenol **6**. This was done to allow activation of the ortho position in compound **6** through inductive and resonance effects.

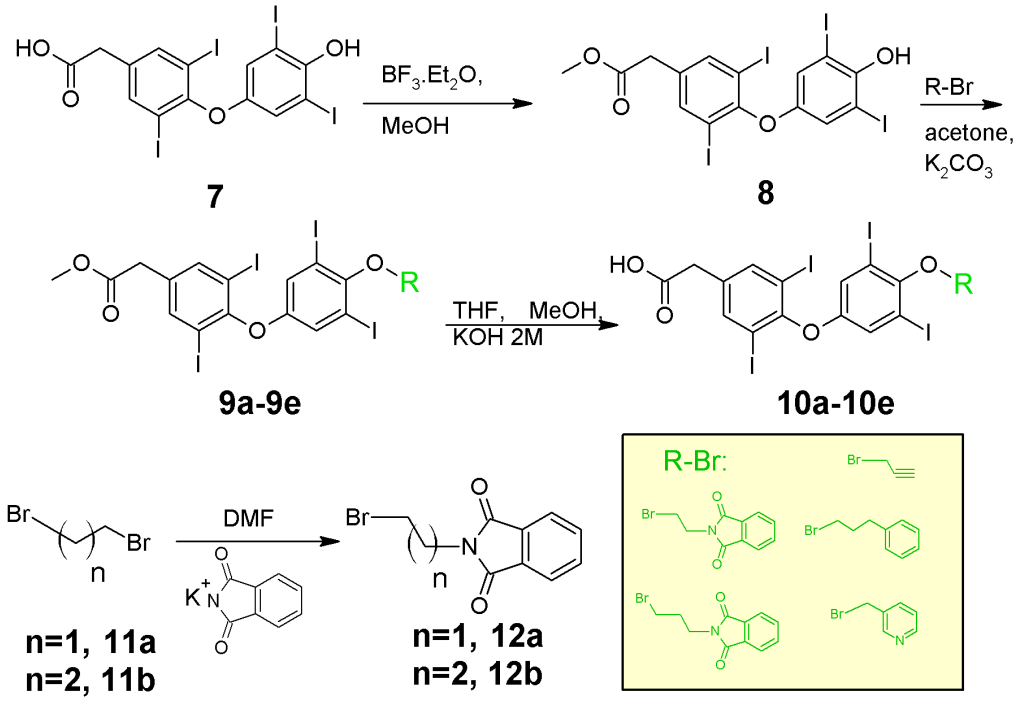


Scheme 1. Synthetic route for the preparation of tetrac, **7**.

The phenolic hydroxyl group of tetrac **7** was selected as the target site for converting tetrac to an integrin antagonist without any changes to its carboxylic acid moiety. In order to increase reaction yields, we initially protected the carboxylic acid moiety of **7**, which was deprotected in the last step because the carboxylic acid moiety is crucial for the thyroid antagonist activity-related bindings and for initial receptor and metal binding as observed in molecular simulations.[25] We then evaluated anticancer activity of those analogs containing carboxylic acids that remained intact.

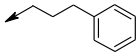
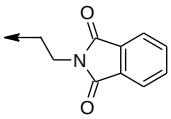
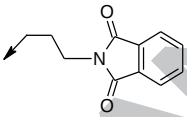
An outline of the synthetic route for the tetrac analogs is shown in **Scheme 2**. The phenyl acetic acid end of tetrac **7** was readily protected to methyl ester using a previously published protecting method,[20] by dissolving **7** and boron tri-fluoride diethyl etherate ($BF_3.Et_2O$) in

methanol. After 24 h stirring in dry conditions at room temperature, saturated $NaHCO_3$ solution was added to quench the reaction and compound **8** (methyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate) was obtained in 62% yield. Further derivatization was provided by reacting the -OH group of **8** with different alkyl bromides in the presence of potassium carbonate and acetone under reflux conditions to give compounds **9a-9e** with yields ranging from 62-98%.[26] The use of potassium carbonate as mild base was critical because amine bases resulted in loss of the iodine in the ortho position of the phenol ring of **8** (outer-ring).[20] A 3-fold excess of alkyl bromides was used in reaction of **8** to **9**, except propargyl bromide (PB), where an excess of PB resulted in transesterification. A 1:1 ratio of PB to **8** yielded 72% of **9a**. Eighty-two percent of **9b** and 62% of **9c** were obtained by reaction of **8** with commercially available 1-bromo-3-phenylpropane and 3-(bromomethyl)pyridine, respectively. It was surprising that more than 95% of **9d** and **9e** was obtained by reaction of **8** with N-(2-bromoethyl) phthalimide **12a** and N-(2-bromopropyl) phthalimide **12b,** respectively. Although **12a** and **12b** are commercially available, we synthesized them from ethylene dibromide **11a** and propylene dibromide **11b**, respectively, in reaction with phthalimide potassium in DMF and yielded 65-75%. Compounds **9a-9e** were finally deprotected in the presence of potassium hydroxide (2 M) and tetrahydrofuran and methanol (1:1) as a co-solvent, yielding the compounds **10a-10e** in yields ranging from 66-87% (**Scheme 2**, **Table 1**).



Scheme 2. Synthetic route for the preparation of tetrac analogs.

Table 1. Target tetrac analogs

Compound	R	Yield (%)
7 (tetrac)	H	91
10a		76
10b		69
10c		66
10d		83
10e		87

To investigate the structure-activity relationships, compounds **10a-10e** were tested for their ability to block angiogenesis in comparison to tetrac. Inhibition of angiogenesis was measured in an *in vivo* study with an assay that exposed test angiogenesis-inducing and inhibitory compounds to cold liquid Matrigel® Matrix (Corning, New York, NY), a mix of several proteins that, after subcutaneous injection into mice, solidified and permitted the formation of new blood vessels.

Female C57/B6 mice aged 5-6 weeks, with body weights of 20 g, were purchased from Taconic Farms (Hudson, NY). All animal studies were conducted at the animal facility of the Veteran Affairs Medical Center (Albany, NY) in accordance with and approved by the IACUC of the Veterans Affairs Medical Center and their current institutional guidelines for humane animal treatment. Animals were maintained under specific pathogen-free conditions and housed 4 animals per cage, under controlled conditions of temperature (20-24°C) and humidity (60-70%) and a 12 h light/dark cycle. Water and food were provided *ad libitum*. Mice were allowed to acclimatize for 5 d prior to the start of experiments. Mice were dived into 7 groups (3 mice per group): control, tetrac, and 5 tetrac derivatives.

Compounds were added at 10 µg/10 µL to 200 µL Matrigel, which contains murine vascular growth factors, and injected subcutaneously in the mice, and the control group received Matrigel

only at 200 µL+ 10 µL vehicle/implant. There were four subcutaneous implants per mouse (2 implants on the right and 2 implants on left backsides of the animal). The Matrigel liquid plug at 4°C became a solid matrix at 37°C for the sustained release of the compounds over 14 days. At day 14 post-Matrigel implant, all animals were sacrificed and vessel formation was quantified by measuring the hemoglobin concentration in the Matrigel plug according to the Drabkin method[27] using spectrophotometry. Briefly, Matrigel plugs were placed into a 0.5 mL tube containing double distilled water and then homogenized for 5-10 min. The samples were centrifuged at 1700 *x g* for 10 min and the supernatants collected. Fifty µL of supernatant was mixed with 50 µL of Drabkin's reagent and allowed to sit at room temperature for 15-30 min, after which it was placed in a 96-well plate and absorbance measured at 540 nm with a Microplate Manager ELISA reader. Hb concentration was expressed as mg/dL based on comparison with a standard curve.

As shown in **Figure 2**, in the Matrigel plug experiment, tetrac and the 5 derivatives significantly inhibited angiogenesis and results are presented as mean ± standard error means (S.E.M.) or ± standard deviation (S.D.) as given in Figure legends. Control and experimental groups were compared and statistically analyzed with ANOVA and student's t-test using SigmaPlot 10.0 software. Differences between control and experimental end points were considered statistically significant if $P < 0.01$. Tetrac analog compounds also blocked the FGF-induced angiogenesis in a comparable fashion to tetrac. Any modulations on either or both sides of tetrac did not greatly affect the antagonist effect of the parent compound. Anti-angiogenesis activity of **10a** might be related to the presence of the alkyne function group on its structure, which may be modified in our further structure-activity studies. Also, anti-angiogenesis activity of compounds **10d** and **10e** were very interesting because of the presence of the phthalimide portion of their structures, which made them similar to thalidomide (**Figure 3**), a known anti-angiogenesis inhibitor that can inhibit angiogenesis induced by angiogenic cytokines such as basic fibroblast growth factor (bFGF) in the rabbit cornea and VEGF in the corneas of mice.[28-30]

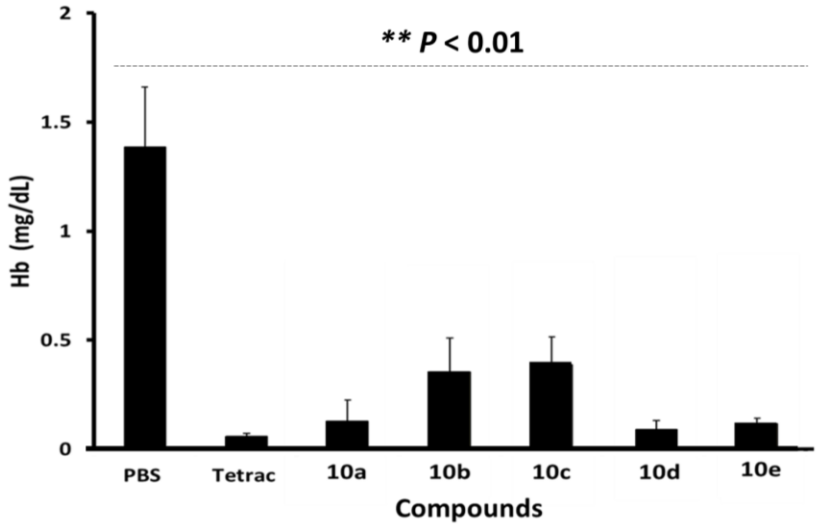


Figure 2. Matrigel plug assay of angiogenesis (anti-angiogenesis effect of tetrac and its analogs). Values are expressed as mean ± SEM versus control vehicle (PBS).

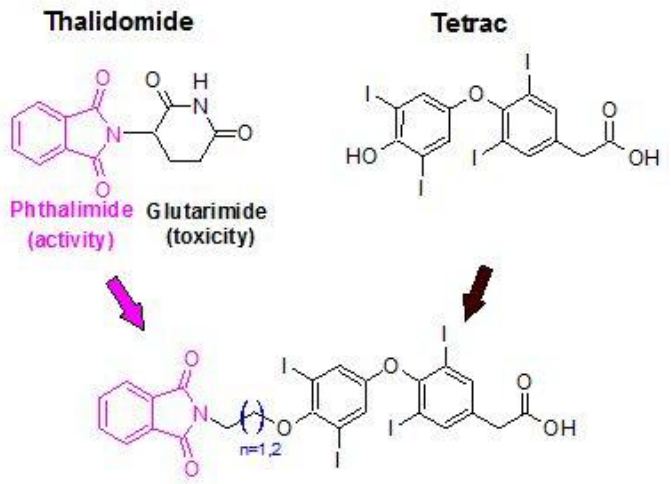


Figure 3. Phthalimide ring of thalidomide is responsible for its activity.

In summary, to find new angiogenesis inhibitors we synthesized new tetrac analogs by modifying the phenolic moiety of tetrac. We analyzed their structure-activity relationships and tested for anti-angiogenesis activity using a mouse Matrigel model for angiogenesis. Design and development of novel angiogenesis inhibitors has been validated as a target in several tumor types. Our results showed that phthalimide-modified tetrac analogs **10d** and **10e** have higher anti-angiogenesis activity than the other analogs generated and this might be due to the presence of the phthalimide portion of their structure, similar to thalidomide. Our further studies will

require additional bioanalytical studies (LC/MS/MS) to see the exact contribution of the phthalimide portion to the *in vivo* activity in the tetrac combination. In order to increase the water solubility of these new materials, we also plan to conjugate these anti-angiogenesis molecules to PLGA-based NPs.

Declarations of interest

S.A.M. holds stock in NanoPharmaceuticals LLC, which is developing anticancer drugs. All other authors declare no competing financial interest.

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Supplementary data

Supplementary data contains information on the synthetic protocols and analytical characterizations of all molecules. Also included are the NMR spectra of some of the synthesized compounds.

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26. General procedure for preparation of 9a-9n: A mixture of methyl protected tetrac 2 (1 eq) and alkyl bromide (2-3 eq) and potassium carbonate in (5 eq) in 25 mL acetone was heated at reflux for 24 h. Reaction was filtered, concentrated, and then crude purified with flash column chromatography over silica gel using n-hexane and ethyl acetate (9:1 to 7:3) to give 3a-3n with 60-80% yield. N-hexane and ethyl acetate (9:1 to 7:3) were used as solvents for TLC.
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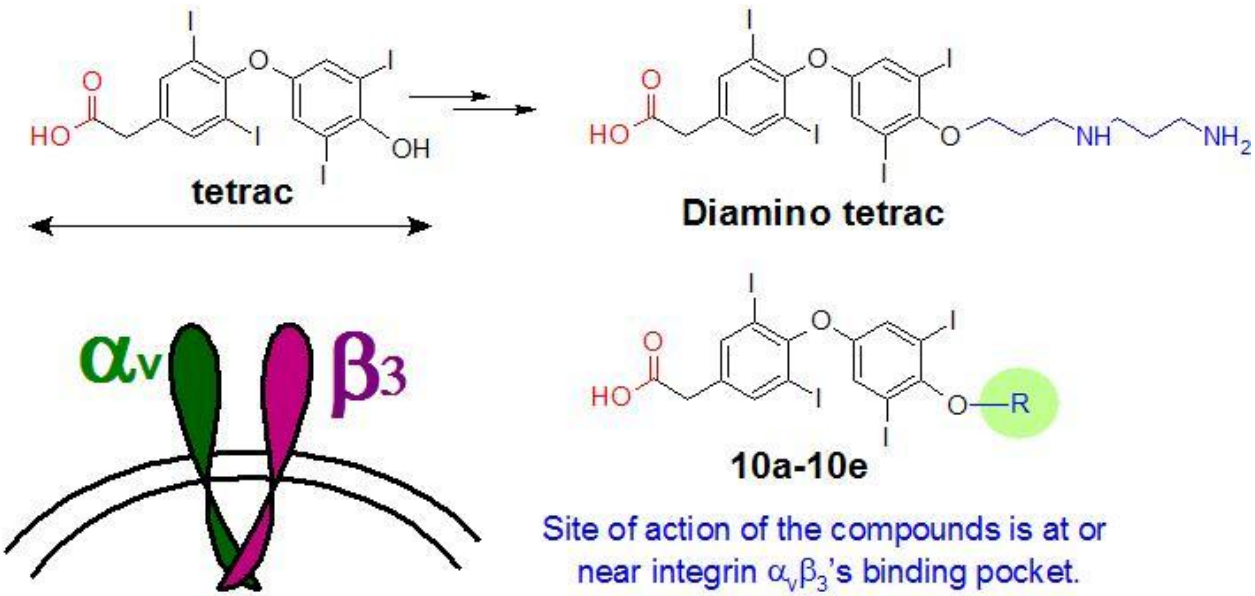
Graphical Abstract



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