5-2017

Design, Synthesis and Characterization of New Analogs of Tetraiodothyroacetic Acid (Tetrac) as Novel Angiogenesis Modulators and their Binding Studies with αvβ3 Integrin

Mary Adeyeye
University at Albany, State University of New York

Design, Synthesis and Characterization of New Analogs of Tetraiodothyroacetic acid (Tetrac) as Novel Angiogenesis Modulators and their Binding Studies with $\alpha_v\beta_3$ Integrin

An honors thesis presented to the
Department of Chemistry,
University at Albany, State University of New York
in partial fulfillment of the requirements
for graduation with Honors in Chemistry
and
graduation from The Honors College.

Mary Adeyeye
Research Mentor: Dr. Shaker Mousa and Dr. Mehdi Rajabi
Research Advisor: Dr. Paul Toscano

May, 2017

Abstract

Angiogenesis is the formation of new blood vessels from preexisting blood vessels. Design and development of novel angiogenesis inhibitors has been validated as a target in several tumor types. Integrins are important ligands that play a critical role in the angiogenesis process, particularly blood formation and local release of vascular growth factors, and they are members of a family of cell surface receptors. Tetraiodothyroacetic acid (tetrac), a deaminated derivative of thyroid hormone **T_4**, is a thyroid antagonist and blocks the actions of **T_3** and **T_4** with an interaction site that is located at or near the **RGD** recognition site identified on integrin **$\alpha_v\beta_3$**'s binding pocket. In this study, we synthesized novel mono- and di-amino tetrac analogs and then tested them for their anti-angiogenesis activity.

Acknowledgments

I would like to thank my research mentors Dr. Shaker Mousa and Dr. Mehdi Rajabi for all their guidance in this process and the opportunity to conduct research at this level. I also wanted to thank my research advisor Dr. Paul Toscano for keeping me focused during this process. I also want to thank my family especially my mom for giving me a good example to look up to during this journey.

Table of Contents

Abstract……....2
Acknowledgments……3
Introduction……..4
General Chemistry and Materials……...12
Results and Discussion……...13
Conclusion…….24
References……..26

Introduction

Judah Folkman in 1971, theorized that tumors cannot grow past a size of 1 mm without developing their own blood supply. [1] Angiogenesis refers to the "process by which resident endothelial cells of the wound's adjacent mature vascular network proliferate, migrate and remodel into neovessels that grow into the initially avascular wound tissue aided by mature stromal cells." [2] In other words, it is the formation of new microvessels from an already established vascular network, expanding the already existing network [3]. Similar but still unlike angiogenesis, vasculogenesis is the differentiation of precursor cells (angioblasts) into endothelial cells and the formation of a primitive vascular network. Vasculogenesis is creating a brand new network, while angiogenesis adds to a pre-existing one [4,5].

Angiogenesis occurs by two distinct mechanisms, sprouting and intussusception. Intussusceptive angiogenesis begins due to the insertion of interstitial cellular columns into the lumen of pre-existing vessels. The growth that follows these columns and their stabilization results in partitioning of the vessel and remodeling of the local vascular network. Sprouting angiogenesis entails two successive phases: neovessel growth and neovessel stabilization. Both phases are equally important because, in the absence of vessel stabilization, the immature capillary will rapidly undergo apoptosis and regress [6].

Tumor angiogenesis is a process of new blood vessel growth of tumors from preexisting vessels by cell adhesion to the extracellular matrix (ECM) and allows for tumor progression. [7] Rapid tumor cell proliferation produces environmental stresses such as a hypoxic, glucose-deprived environment that begins the angiogenic switch, whereby tumor cells initiate to spatter proangiogenic growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), angiopoietin, and thrombospondin **(Figure 1)**

[8-10].

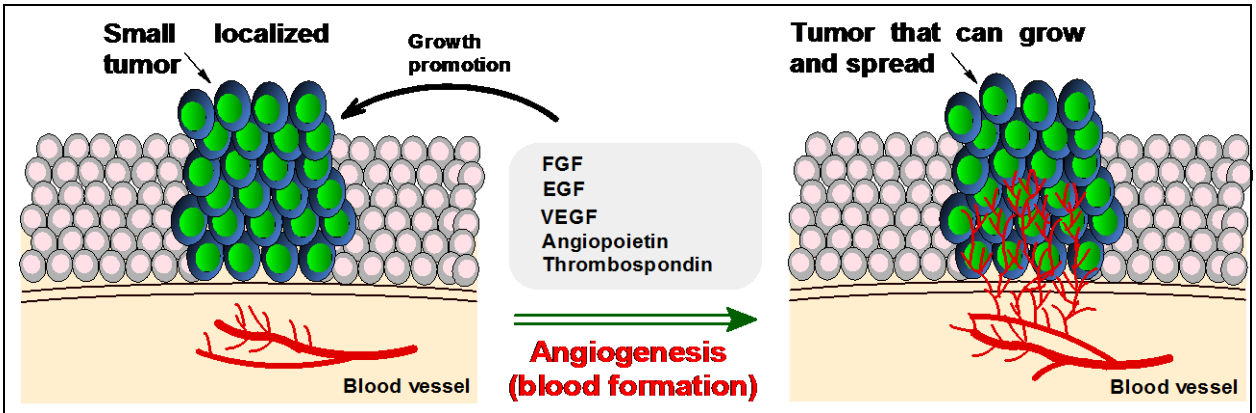


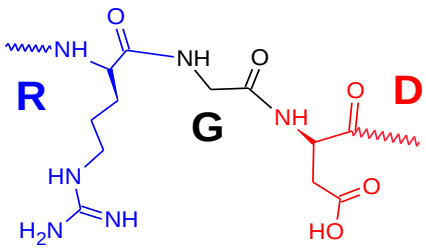
Figure 1. Angiogenesis is the process of the development of new blood vessels from preexisting vessels, which allows for tumor progression. The tumor starts off localized but with growth factors like FGF, EGF, VEGF, angiopoietin and thrombospondin, the blood vessel is encouraged to grow and spread into the tumor. This allows the tumor to grow and spread with the additional blood vessels inside the tumor, giving the tumor access to the rest of the body.

One of the important ligands that play a critical role in the angiogenesis process particularly blood formation and local release of vascular growth factors, are integrins. They are members of a family of cell surface receptors who are immunoglobulin superfamily molecules or ECM proteins [11-13]. The integrins consist of α and β chain units including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_4\beta_1$, $\alpha_5\beta_4$, and $\alpha_6\beta_4$ are expressed on multiple tumor types (**Table 1**).

Table 1. Selected list of integrins in cancer progression

Integrins expressed	**Tumor type**
$\alpha_v\beta_3$	Glioblastoma [14], cervical [15], ovarian [16], pancreatic [17], prostate [18], breast [19-22], and melanoma [23-24]
$\alpha_v\beta_5$	Glioblastoma [14]
$\alpha_v\beta_6$	Colon [25] and cervical [26]
$\alpha_4\beta_1$	Ovarian [27]
$\alpha_5\beta_1$	Non-small-cell lung cancer [28] and melanoma [24]
$\alpha_6\beta_4$	Breast [30-31]

Among them, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are the main receptor types involved in the angiogenesis process, particularly in binding with angiogenesis modulators containing arginine-glycine-aspartate (RGD) that bind to specific recognition site of integrin receptor (identified as the RGD recognition site). [32-34] There are several natural RGD containing proteins such as fibronectin, fibrinogen, λ-receptor on *E. coli*, Sindbis coat protein, and α-lytic protease protein as well as cyclic RGD tripeptide (c-RGD) and c-RGD peptidomimetics that have shown high binding affinity to the recognition site of the integrin **(Figure 2)** [35-37]. Artificial RGD-containing peptides are either cyclic or linear RGD peptides**.** Cyclic RGD peptides are more active than linear RGD peptides because they are conformationally less flexible and metabolically more stable. [38]



Proteins	Sequence
Fibronectin	AVTG**RGD**SPASSK
Fibrinogen	TSYN**RGD**STFESK
γ-receptor on E.coli	GSFG**RGD**SDEWTF
Sindbis coat protein	GVGG**RGD**SGRPIM
α-lytic protease	ACMG**RGD**SGGSWI

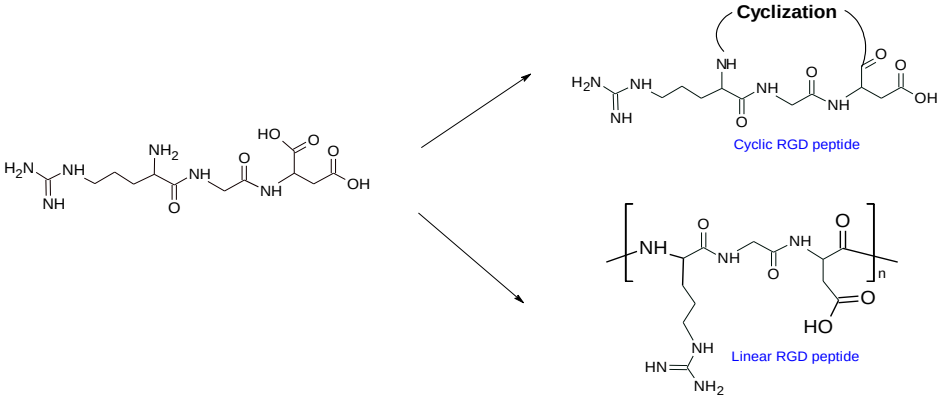


Figure 2. Chemical structure of arginine-glycine-aspartate (RGD) and some known natural RGD containing proteins.

RGD peptides attach to the integrin $\alpha_v\beta_3$; this integrin is important in angiogenesis and is

expressed highly in tumor cells. [39] The $\alpha_v\beta_3$ antagonist inhibits tumor angiogenesis. Due to the small size of the RGD peptides, RGD conjugates have easier access to tumor tissues **(Figure 2)**. Several studies have reported the effects of thyroid hormone derivatives on thyroid hormone receptor (TR) and membrane Na^+/H^+ antiporter ion pumps via binding to integrin $\alpha_V\beta_3$. For example, angiogenesis activity of thyroid hormone derivatives has been observed with 2-ammonium-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]-propionate (T_3) and 2-ammonium-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl] propionate (T_4), and anti-angiogenesis activity has been observed with tetraiodothyroacetic acid (tetrac) in the chick chorioallantoic membrane (CAM) and other angiogenesis models. [40-43] Tetrac is a deaminated derivative of T_4 and acts as a thyroid antagonist by blocking the actions of T_3 and T_4 with an interaction site that is located at or near the RGD recognition site on integrin $\alpha_v\beta_3$'s binding pocket. **(Figure 3A)** Tetrac also showed anti-proliferative activity against different cancer cell lines such as human non-small cell lung cancer (NSCLC) H1299 cells *in vitro* and in xenografts as well as anti-angiogenic activity at the integrin $\alpha_v\beta_3$ receptor binding site. [44] For example, Yoshida *et al.* found tetrac to be an effective inhibitor of retinal angiogenesis and was able to inhibit the pro-angiogenic effect of both VEGF and EPO on retinal endothelial cells; this suggests that tetrac, an antagonism of integrin $\alpha_v\beta_3$, is a viable therapeutic strategy for proliferative diabetic retinopathy [45].

Our group widely uses tetrac for conjugation to the polymeric nanoparticle (NP) poly(lactic-co-glycolic acid) (PLGA) *via* covalent binding (an amidation reaction) between tetrac's outer ring hydroxyl to NHS-modified PLGA, which resulted in a tetrac-nanoparticulate. This nanoparticle showed anti-angiogenic activity, which confirmed the role of the integrin receptor. **(Figure 3B)** [46-48] Results obtained from both *in vitro* and *in vivo* experiments for the treatment of drug-

resistant breast cancer showed that tetrac-conjugated PLGA-NPs were restricted from entering into the cell nucleus and enhanced inhibition of tumor-cell proliferation at a low-dose equivalent of free tetrac. We concluded that tetrac-conjugated PLGA-NPs have a high potential as anticancer agents, with possible applications in the treatment of drug-resistant cancer [47, 49-52]. We also synthesized a new analog of tetrac that expresses pro-angiogenic rather than anti-angiogenic activity. In order to mimic the action of the iodothyronine deiodinases (the enzymes that convert T_4 to T_3), tetrac was deiodinated using tertiary amines. Tertiary amines initially deprotonated the phenolic OH group 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 [53].

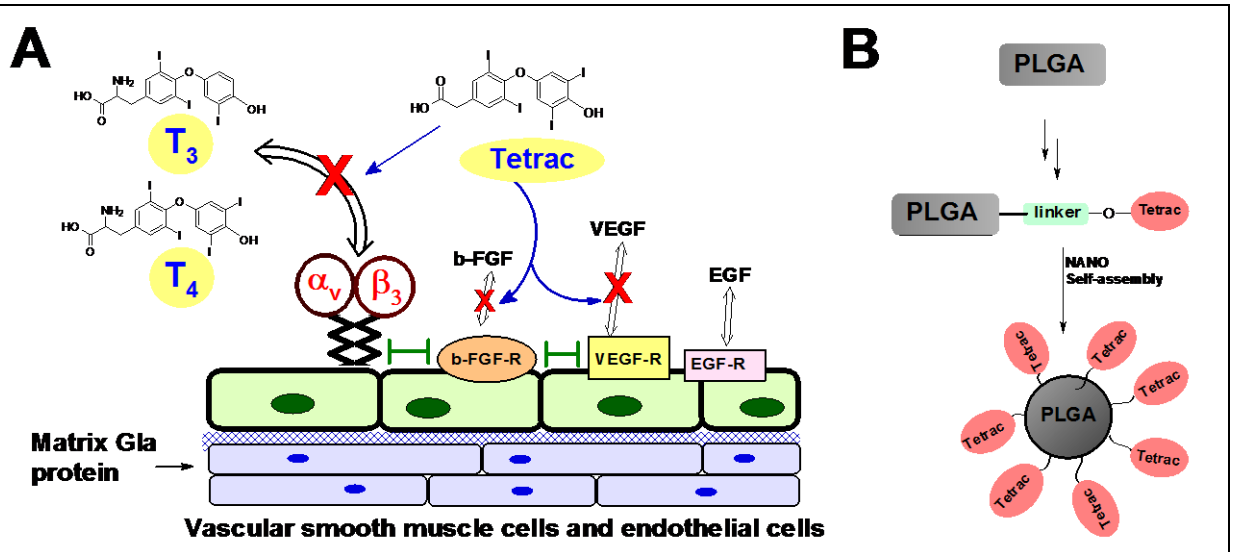


Figure 3. A) Pro-angiogenic actions of thyroid hormones (T_4 and T_3) on endothelial cells and vascular smooth cells are initiated at the cell surface receptor for the hormone on the extracellular domain of integrin $\alpha_v\beta_3$. Tetraiodothyroacetic acid (tetrac), a thyroid hormone analog, is inhibitory at the integrin receptor and is anti-angiogenic. **B)** Conjugation of tetrac to a polymeric nanoparticle like poly(lactic-co-glycolic acid) (PLGA) results in a tetrac-nanoparticulate that has shown anti-angiogenic activity.

Knowing that the angiogenesis activity of tetrac is stimulated by VEGF or FGF without

influencing the pre-existing blood vessels, we planned to synthesize new analogs of tetrac by modifying the phenolic moiety of tetrac and study their structure-activity relationship. The phenolic hydroxyl group (-OH) of thyroid analogs is an important site for modification and a target site for converting tetrac to an integrin antagonist without any changes to the carboxylic acid moiety of tetrac. As an example, we previously synthesized diamino-tetrac (DAT), which showed anticancer/anti-angiogenic activity that was able to target the thyroid hormone-tetrac receptor on the extracellular domain of integrin $\alpha_v\beta_3$. **(Figure 4A)**

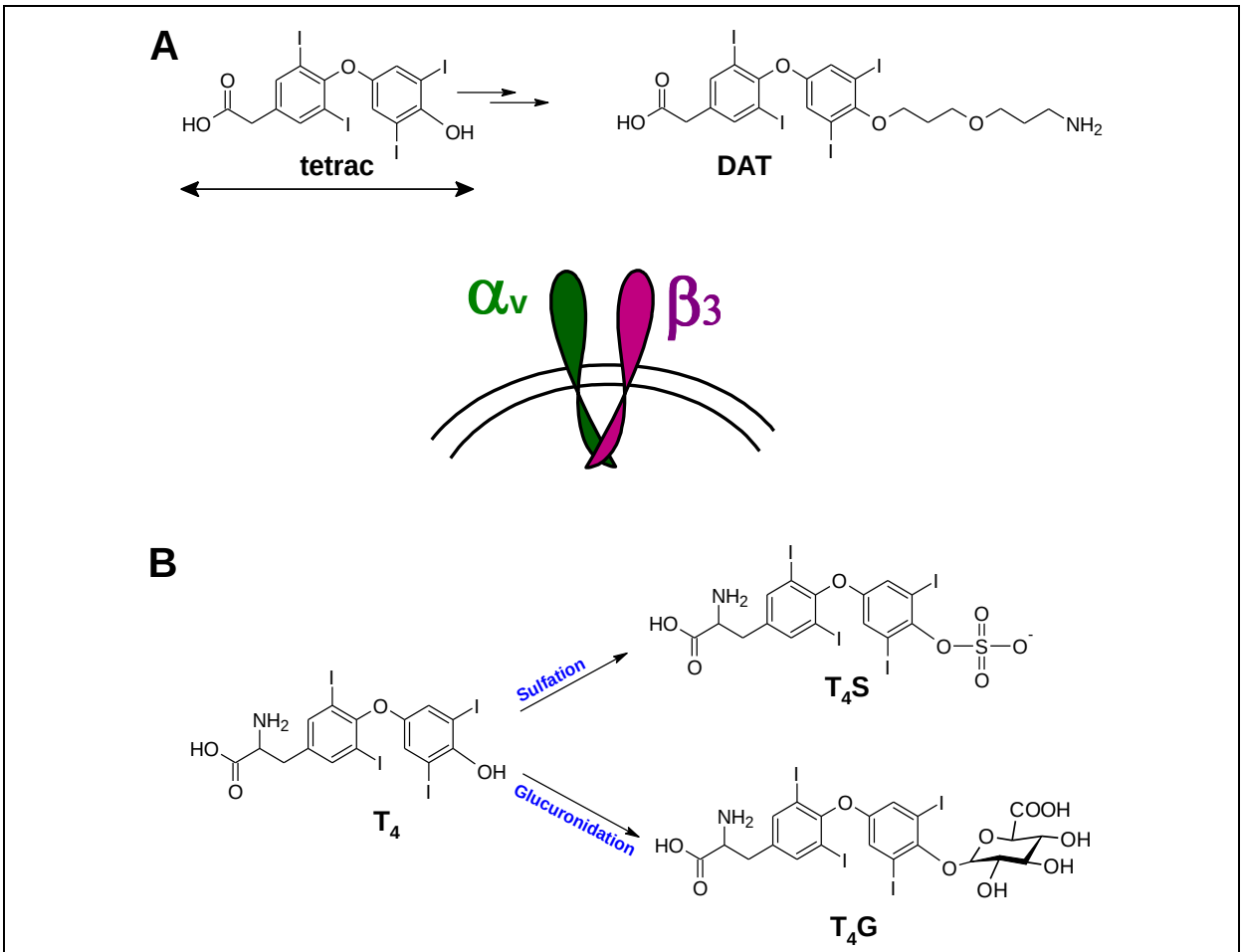


Figure 4. A) The phenolic hydroxyl group (-OH) of tetrac is selected as the modification site and target site for converting tetrac to an integrin antagonist without any changes to the carboxylic acid moiety of tetrac. **B)** Conjugation of the phenolic hydroxy group (4'-OH) of thyroxine (T_4) with sulfate or glucuronic acid yields the corresponding sulfated (T_4S) and glucuronidated hormone (T_4G), respectively.

Wu *et al.*, also showed that conjugation of the phenolic hydroxy group (4'-OH) of thyroxine (T_4) with sulfate or glucuronic acid yielded the corresponding sulfated (T_4S) and glucuronidated (T_4G) hormone, respectively; sulfate and glucuronic acid conjugations are useful in enhancing the water solubility of many hydrophobic drugs and their excretion through urine and/or bile. [54-55] Similar to T_4S and T_4G, tetrac also undergoes sulfation and glucuronidation, thus suggesting that

the body can integrate drugs into metabolism. [55] **(Figure 4B)**

General chemistry and materials

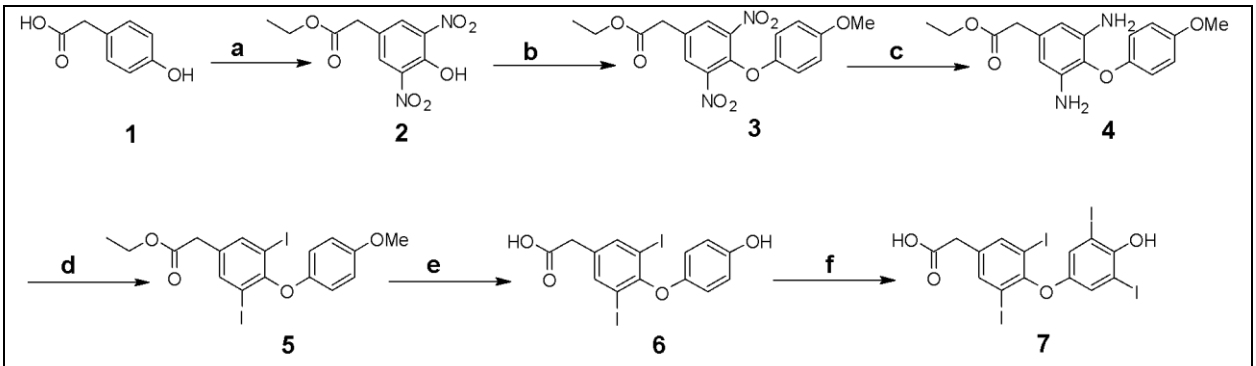
All commercially available chemicals were used without further purification. All solvents were dried and anhydrous solvents were obtained using activated molecular sieves (0.3 or 0.4 nm depending on the type of solvent). All reactions (if not specifically containing water as reactant, solvent or co-solvent) were performed under an Ar or N_2 atmosphere, in oven-dried glassware. All new compounds gave satisfactory ^{1}H NMR and mass spectrometry results. Melting points were determined on an Electrothermal MEL-TEMP® melting point apparatus and on a Thomas HOOVER Uni-mel capillary melting point apparatus. Infrared spectra were recorded on a Thermo Electron Nicolet Avatar 330 FT-IR apparatus. UV spectra were obtained from a SHIMADZU UV-1650PC UV-vis spectrophotometer. The solution-state NMR experiments were all performed on a Bruker Advance II 800 MHz spectrometer equipped with a cryogenically cooled probe (TCI) with z-axis gradients (Bruker BioSpin, Billerica, MA) at the Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute (RPI, Troy, NY). All tubes used had a 5 mm outside diameter. NMR data were referenced to chloroform ($CDCl_3$; 7.27 ppm ^{1}H, 77.20 ppm ^{13}C) or DMSO-d6 (δ= 2.50 ppm, 38.92 ppm ^{13}C) as internal reference. Chemical shifts δ are given in ppm; multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad); coupling constants, J, are reported in Hz. Thin layer chromatography was performed on silica gel plates with a fluorescent indicator. Visualization was accomplished by UV light (254 and/or 365 nm) and/or by staining in ceric ammonium molybdate or sulfuric acid solution. Flash column chromatography was performed following the procedure indicated by Still *et al.*, with 230-400 mesh silica gel. [56] High resolution mass spectral analysis was performed on either an Applied Biosystems API4000 LC/MS/MS or Applied Biosystems QSTAR XL mass

spectrometers.

Results and discussion

Synthetic methods for synthesis of tetrac and its analogs

As shown in **Scheme 1**, a large scale of compound **7**, tetrac, was synthesized according to our previously described method. [57] Compound **2**, (4-hydroxy-3,5-dinitro-phenyl)-acetic acid ethyl ester was obtained from commercially available 4-hydroxy-benzeneacetic acid **1** in the presence of nitric acid, acetic acid, and ethanol in 72% yield, which was then reacted with *p*-toluenesulfonyl chloride and methoxyphenol in pyridine to give the diphenyl ether derivative **3** in 75% yield. Two nitro groups of **3** were reduced and converted to amine **4** using a catalytic amount of 5% palladium, H_2, calcium carbonate and ethanol was the solvent. Compound **4** was then reacted with sodium iodide to convert the amine groups to iodine, resulting in **5** in 69 % yield, followed by hydrolyzation and deprotection under reflux conditions using hydroiodic acid (HI) as a strong cleaving agent for facile removal of both the ester and methyl groups from product **6**. Acetic acid was used to avoid decomposition of HI into I_2 by H_2SO_4, thereby avoiding the generation of unwanted side products in the reaction medium. The tetrac target molecule **7** was obtained from the di-iodo compound **6** by reacting with iodine. In the reaction, iodo-deprotonation was carried out using I_2 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 an alcohol in substrate **6**. This was done to allow activation of the ortho position in compound **6** through inductive and resonance effects.

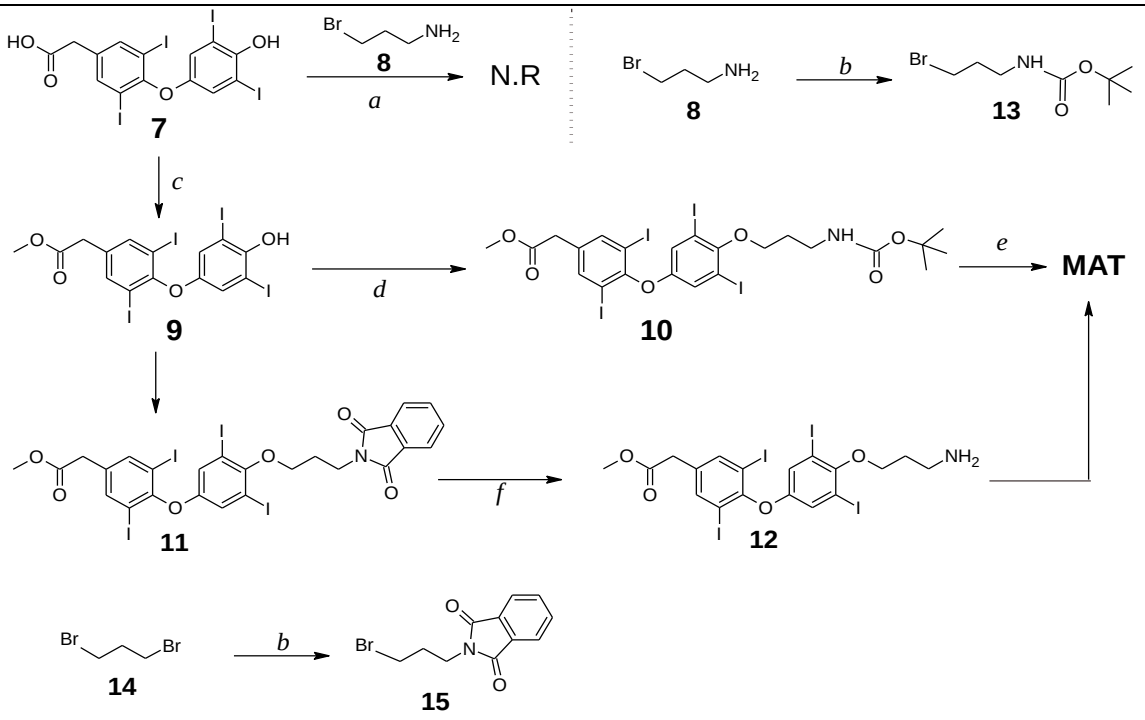


Scheme 1. Preparation of tetrac **7**; **(a)** nitric acid, acetic acid, ethanol, 24 h, 72%; **(b)** *p*-toluenesulfonyl chloride, *p*-methoxyphenol, pyridine, 3 h, 95-125°C, 75%; **(c)** catalytic amount of 5% palladium, H_2, calcium carbonate, ethanol, quantitative yield; **(d)** i) sodium nitrate, sulfuric acid/acetic acid (v/v), 0°C, 2 h, ii) sodium iodide, I_2, urea, water/$CHCl_3$ (3/1), RT, 1 h; **(e)** HI (excess), acetic acid, reflux, 1 h, 95%; **(f)** I_2 excess, NH_3 excess, CH_3OH, 91%.

As mentioned, the phenolic hydroxyl group (-OH) of tetrac was selected as the target site for converting tetrac to an integrin antagonist without any changes of its carboxylic acid moiety. For each new compound the carboxylic acid moiety is mainly responsible for the ligand-receptor interaction, and is involved in the binding of a metal ion (2.6 A from the metal ion) that occurs only in the active state of the integrin receptor. [54] Modification of the hydroxyl group of tetrac with alkyl chains carrying at least one amino group, will shift the antagonist strength of tetrac upward because of their ability to interact with the Arg recognition site in the propeller domain of integrin $\alpha_v\beta_3$. In addition to the number of nitrogen atoms, we strongly believe that the distance between the carboxylic acid and the amine groups will impact the activity of molecule [54,58]. In this study, we synthesized mono-amino tetrac (MAT) from tetrac in different approaches and analyzed its biological activity compared to DAT as well as its binding affinity with $\alpha_v\beta_3$ and molecular modeling [54].

In order to synthesize MAT, we initially began by treating tetrac **7** directly with 3-

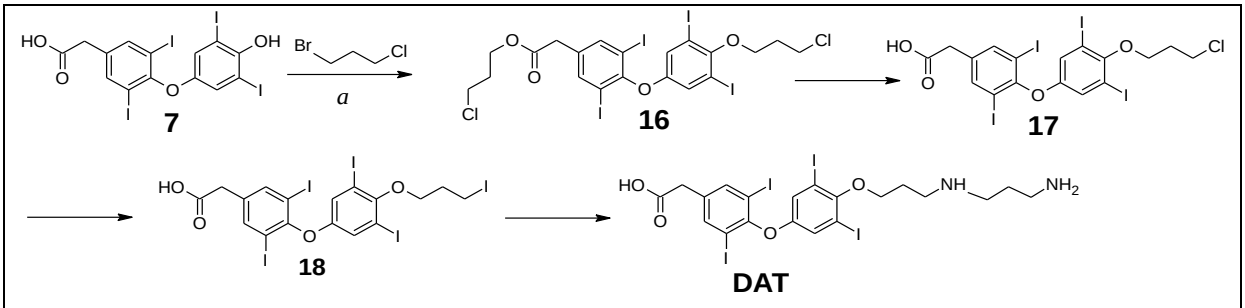
bromopropylamine **8** in different conditions but it did not work. Therefore, we decided to protect the amine group of 3-bromopropylamine **4** with *tert*-butyl carbamate (Boc) using di-tert-butyl dicarbonate (Boc_2O) in the presence of triethylamine (TEA, 4 eq) in dichloromethane for 12 hours giving the desired 3-bromopropyl-tert-butylcarbamate **13** in 96% yield. [60] In order to have better reaction yields, we also protected the phenylacetic acid end of tetrac **7** using a protecting method that we previously published, by dissolving **tetrac 7** and thionyl chloride in methanol under reflux conditions and compound **9** (methyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate) was obtained in 78 % yield. [59] Results from FTIR confirmed the production of protected tetrac **9** by the shift of the carbonyl (C=O) of the acid group of tetrac from 1693 cm^{-1} to 1719 cm^{-1} as evidence of the carbonyl (C=O) of the ester group after protection. This was also confirmed by ^{1}H NMR and ^{13}CNMR data. [59] The -OH of **9** was then reacted with 3-bromopropyl-tert-butylcarbamate **13** in the presence of potassium carbonate and acetone under reflux conditions to give protected compound **10** with a yield of approximately 93%. Using the potassium carbonate as mild base is very important for deprotonation of the phenol and for the reaction with alkyl bromide.



Scheme 2. (a) boron trifluoride diethyl etherate ($BF_3.Et_2O$), methanol, 24 h, dry conditions, room temperature, 62%; **(b)** alkyl bromide, acetone, potassium carbonate, reflux, 18 h, 65-80%; **(c)** THF, MeOH, KOH 2M, 70-90%; **(d)** 3-bromopropyl-tert-butylcarbamate, potassium carbonate and acetone, reflux, 93%; **(e)** concentrated acetic acid, 78% (**f)** NH2NH2, ethanol.

Subsequently, compound **10** was finally deprotected in the presence of concentrated acetic acid. A novel and efficient method to convert fully protected amino acids to its corresponding zwitterionic forms was developed. Design and synthesis of non-natural amino acids requires multiple protecting groups, and final manipulations of synthetic amino acids into their zwitterionic form typically involves isoelectric precipitation or laborious ion exchange chromatography. [61] Based on previously reported work by Hao *et al.*, in order to avoid ion exchange chromatography and obtain zwitterionic amino acids after solvent removal, we employed acetic acid to simultaneously remove both the methyl ester and the Boc protecting groups. [62] Although Hao *et al.*, completed the reaction in microwave conditions at 150 °C, we set the reaction in reflux in

highly concentrated acetic acid. This fully protected tetrac analog **10** was cleanly converted to zwitterion MAT in 78% yield after simple evaporation of solvents (AcOH and water) without any further purification or manipulation.



Scheme 3. Synthesis of DAT (a) potassium carbonate, 1-bromo-3-chloropropane, acetone, reflux; **(b)** concentrated HCl; **(c)** acetone, sodium iodide, reflux, 26h; **(d)** 1, 3-diaminopropane, stirred for 2h, 1N sodium hydroxide

DAT was also synthesized based on our previously reported patent as the reaction shown in **scheme 3** by adding potassium carbonate to a solution of tetrac **7** and 1-bromo-3-chloropropane in acetone that was vigorously mixed to produce a white, thick suspension that was slowly (30 min) heated to a gentle reflux (58-59 °C). [63] After 5.5 hours the reaction mixture was cooled to ~ 45 °C, filtered and washed with dichloromethane. The filtrate was evaporated to oil, to which n-Heptane is added. The white solids of dichloro-tetrac were allowed to stand overnight at room temperature and then filtered. The solids were washed with n-Heptane and dried under vacuum (room temperature). Dichloro-tetrac was brought up in ethanol and added to a solution of potassium hydroxide in ethanol. The resulting mixture was acidified with conc. hydrochloric acid in ethanol to an approximate pH of 1. The resulting suspension was evaporated to reduce the volume by half and deionized water was added. The mixture was cooled to room temperature and then filtered. The precipitate was washed with deionized water and dried at 45 °C under vacuum to yield chloro-tetrac **17**. The chloro-tetrac was dissolved in acetone and added to a solution of

sodium iodide in acetone. The reaction mixture was heated to a gentle reflux (59-60 °C) for 26 h. The reaction mixture was evaporated to remove most of the acetone and the resulting solid was stirred with deionized water for 1 hour. The solids were filtered and washed with deionized water. The solid was dried at 45-50 °C under house vacuum for four days to give iodo-tetrac **18** as a pale yellow solid. Iodo-tetrac was slowly added to 1,3-diaminopropane while maintaining the temperature below 28 °C. The clear reaction mixture was stirred at room temperature for 2 hours. A solution of 1N sodium hydroxide was added and most of the solvent was evaporated at 50-55 °C using a high vacuum pump. The crude diamino-tetrac was purified over silica gel (70-230 mesh) eluting with a mixture of DCM: MeOH: 7N NH3 60:40:6. Pure fractions were collected and evaporated. The resulting mixture was stirred with a 2:3 mixture of ethanol: CH2C12. The solids were filtered and washed with a 1:1 mixture of ethanol:CH2D12. The product was dried at 45 °C overnight to give **DAT**.

In vitro adhesion studies

Adhesion of mammalian cells to the extracellular matrix (ECM) is mediated by protein-protein and protein-carbohydrate interactions. Alterations in the expression of cell surface molecules lead to the dissemination of metastatic cells from tumor tissue. Cell surface molecules in glioma, which are important for their metastatic property, have been intensively investigated. Among these, integrins, which are heterodimeric integral membrane proteins, are involved in protein-protein mediated adhesion of cells to the extracellular matrix (ECM).

Adhesion assay

The 96-well plates polyvinyl chloride (PVC) were coated with 0.5 μg human fibrinogen dissolved in 50 μl H_2O and incubated overnight at 4 ^{0}C. Nonspecific binding sites were blocked with 200 μl of 2 % bovine serum albumin (BSA) solution in phosphate buffer saline (PBS). To

control for nonspecific adherence to the PVC surface, cell adhesion was also measured on non-coated PVC plates. Glioma (U87) cells were detached with 0.05 % Trypsin EDTA and washed three times in assay-medium (serum-free RPMI medium). Test compounds (MAT, DAT and tetrac) were dissolved in assay medium and cells were incubated therein for 1 h. For the adhesion assays on PVC, the assay medium contained no BSA. To each coated well 1 X 10^5 cells were added, and after 1h nonadherent cells were removed by three consecutive washing steps with PBS. Cells were fixed with 4 % paraformaldehyde and stained in 0.5 % crystal violet in 20 % MeOH/PBS. Cells were washed extensively with H_2O, solubilized with 1 % SDS, and the absorbance read was at 590 nm.

Inhibition of glioblastoma cell adhesion by compounds

Our cell adhesion studies showed the compounds have similar inhibition of glioma cell attachment to fibrinogen which was used as a control multifunctional adhesive protein that plays a major role in cellular events such as thrombosis, wound healing, and cancer. Studies have demonstrated that the β_3 integrins, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, serve as receptors for fibrinogen on various nucleated cells and platelets, respectively. Therefore, it will be interesting to understand the molecular basis of cell adhesion of synthesized in comparison to fibrinogen. To find the possible binding sites for tetrac, MAT and DAT, we used docking studies to screen the protein surface of the extracellular domain of $\alpha_v\beta_3$, the crystal structure. Inhibition of adhesion of the U87 cells to fibrinogen were tested with increasing concentration of tetrac, MAT and DAT. The cells were exposed to the compounds in medium for 1h, then aliquots of the medium with 1X 10^5 cells were seeded into a well precoated with fibrinogen and incubated for another hour. Non-adherent cells were washed off and adherent cells were quantified at 590 nm. Means of three values ± SD relative to cells adhering in the absence of compounds are shown. The cell adhesion to fibrinogen decreased

significantly (***P < 0.01) in a dose-dependent manner in the presence of compounds until cell binding completely blocked at 1 µM concentration. The inhibition of U87 cells to fibrinogen follows in the order of MAT >DAT > Tetrac at 0.01, 0.03, 0.1, 0.3 and 1 µM concentration. No significance was observed for the binding of tetrac to fibrinogen when compared to cells without any compounds. (**Figure 5)**

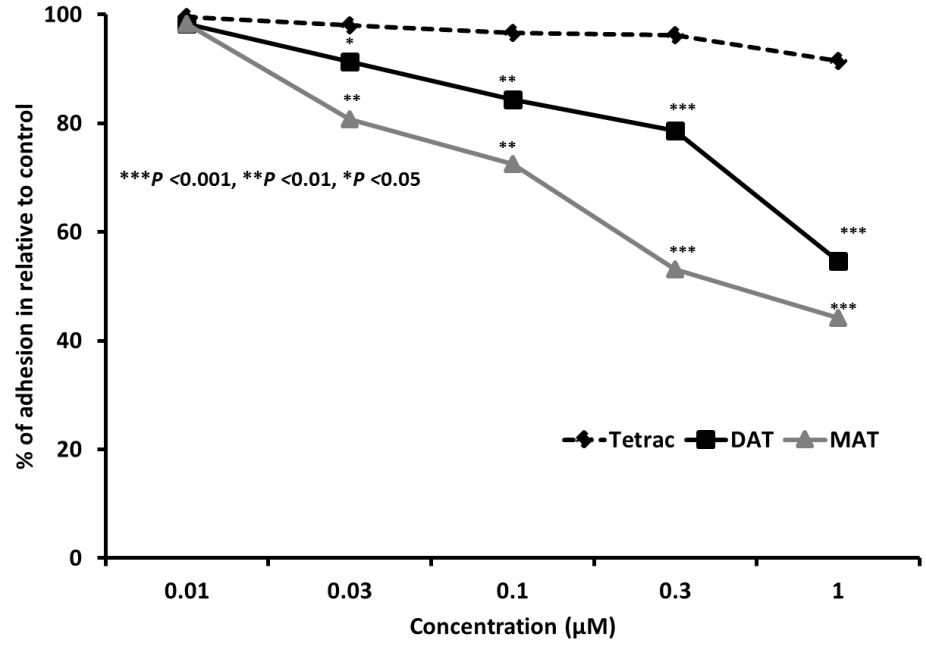


Fig 5: Inhibition of U87 adhesion to fibrinogen. Means of three values ± SD relative to cells adhering in the absence of compounds are shown. ***P <0.001, **P <0.01, *P <0.05.

In silico

Drugs were screened against integrin $\alpha_v\beta_3$ by using Auto Dock Vina, software available from http://viba.scripps.edu/. The crystal structure of integrin $\alpha_v\beta_3$ (PDB id: 1L5G) was retrieved from the Protein Data Bank and the ligand 3D structure were saved in pdbqt format. The grid size along the x, y, z axis was set to 50, 50, 50 Å and the grid center along the x, y, z axis was set as 28.98, 25.32, 19.34 Å, respectively to cover the protein. The interactions of integrin $\alpha_v\beta_3$ with the

drugs, hydrogen bonds and bond lengths were analyzed using pyMOL software**.**

In silico studies

Auto Dock Vina version 4.0 was employed for the docking of drugs to integrin $\alpha_v\beta_3$ protein and the binding affinities of docked compound were obtained. The binding affinity, number of hydrogen bonds formed and catalytic site residues involved in the complex is shown in Table 2 and **Figure 6 (a-c).**

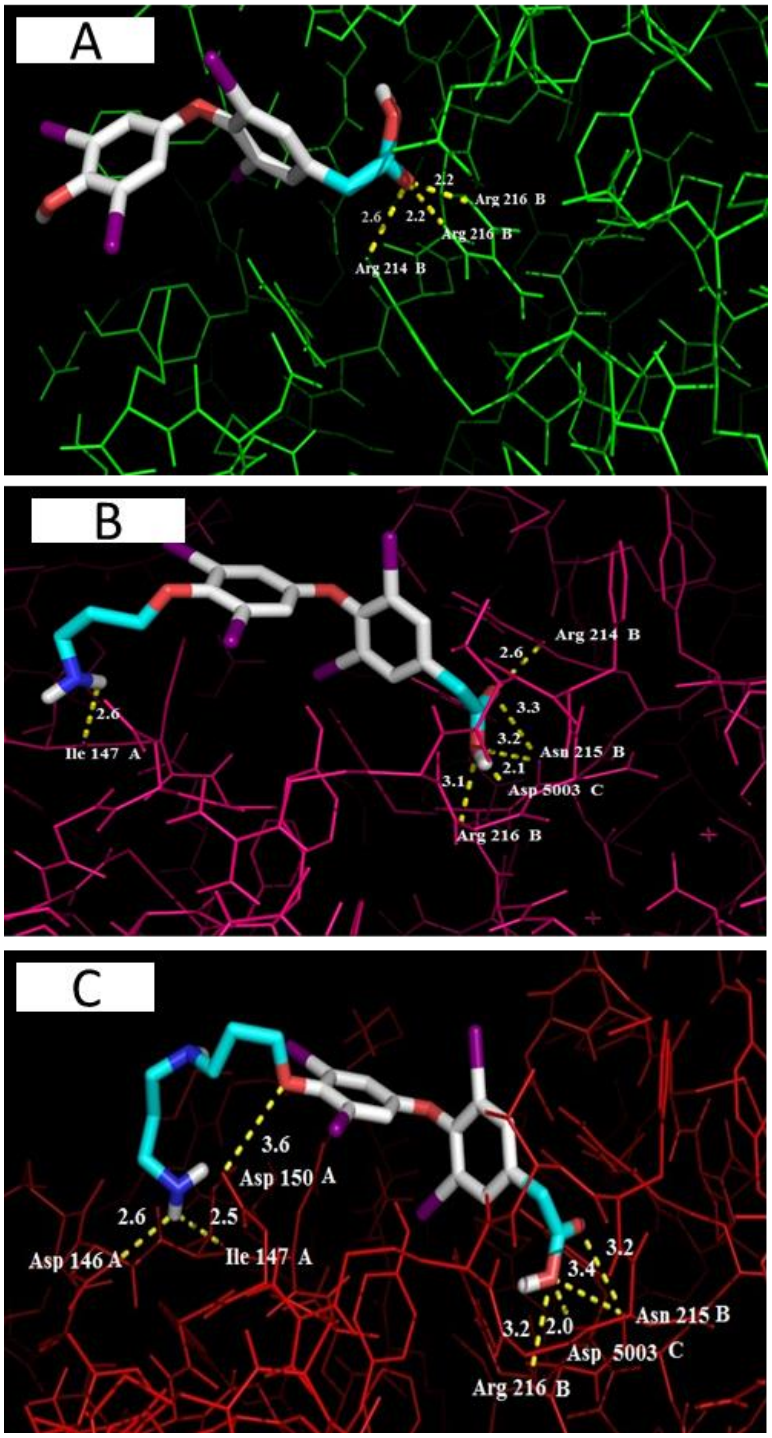


Figure 6. Tetrac , MAT, DAT docked in the crystal structure of the extracellular domain of $\alpha_v\beta_3$ integrin

The tetrac **(Figure 6A)** exhibited binding score of -4.8 Kcal mol^{-1} and resulted in formation

of three hydrogen bonds with amino acids Arg 214 (2.6 Å), Arg 216 (2.2 Å), Arg 216 (2.2 Å) of chain B. Whereas, the MAT **(Figure 6B)** exhibited binding score of -5.4 Kcal mol^{-1} and resulted in formation of six hydrogen bonds with amino acids residues Ile 147 (2.6 Å) of chain A and Arg 214 (2.6 Å), Asn 215 (3.3 Å), Arg 215 (3.2 Å), Arg 216 (3.1 Å) of chain B and Asp 5003 (2.1 Å) of chain C. The diamino-tetrac (**Figure 6C**) exhibited binding score of -5.1 Kcal mol^{-1} and resulted in formation of seven hydrogen bonds with amino acids Asp 146 (2.6 Å), ile 147 (2.5 Å), Asp 150 (3.6 Å) of chain A and Asn 215 (3.2 Å), Arg 215 (3.4 Å), Arg 216 (3.2 Å) of chain B and Asp 5003 (2.0 Å) of chain C. The docking results suggest that the MAT has a higher affinity to bind to the protein compared to diamino tetrac and tetrac.

Table 2. Virtual screening of drugs docked to Integrin $\alpha_v\beta_3$ (IL5G)

Compound Name	Binding Energy (kcal mol^{-1})	No. of Hydrogen bonds	Residues involved in hydrogen bond (Distance (A^0))		
Tetrac	-4.8	3	Chain A (αV)	Chain B (β3)	Chain C
				Arg 214 (2.6) Arg 216 (2.2) Arg 216(2.2)	
Mono amino Tetrac	-5.4	6	Ile 147(2.6)	Arg 214(2.6) Asn 215(3.3) Asn 215(3.2) Arg 216(3.1)	Asp 5003 (2.1)
Diamino tetrac	-5.1	7	Asp 146(2.6) Asp 150(3.6) Ile 147 (2.5)	Asn 215 (3.2) Asn 215 (3.4) Arg 216 (3.2)	Asp 5003 (2.0)

***In vivo* Mouse matrigel model of angiogenesis**

Matrigel plug assays in mice were performed in accordance with institutional guidelines for animal safety and welfare. Female C57/B6 mice aged 5-6 weeks and body weights of 20 g were purchased from Taconic Farms (Hudson, NY, USA). Animals were maintained under specific pathogen-free conditions and housed four 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. The *in vivo* study was carried out in the animal facility of the Veterans Affairs (VA) Medical Center, Albany, NY, and the experimental protocol was approved by the VAIACUC. Mice were allowed to acclimatize for 5 d prior to the start of experiments. Matrigel Matrix High Concentration with growth factors was injected in four subcutaneous ways for each animal at 200 µl/animal. Animals in the control group were injected just with matrigel in 200 µl volume. Animals in the groups were injected with 20 µg compound in 200 µl matrigel. All groups had three mice per group, with a total of 12 matrigel subcutaneous injection per group. At day 14 post plug implant all animals were sacrificed and hemoglobin contents were quantitated using spectrophotometry.

Determination of Hb levels

Matrigel plug Hb content was indexed as a measure of new vascularity. 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 subjected to centrifugation at 4,000 rpm for 10 min and then the supernatants were collected. A volume of 50 µl of supernatant were mixed with 50 µl of Drabkin's reagent and allowed to sit at room temperature for 15-30 min, after which 100 µl 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/ml based on comparison with a standard curve. Figure 7 showed that tetrac had a higher activity when compared to MAT and DAT but when compared to

DAT, MAT had a higher activity.

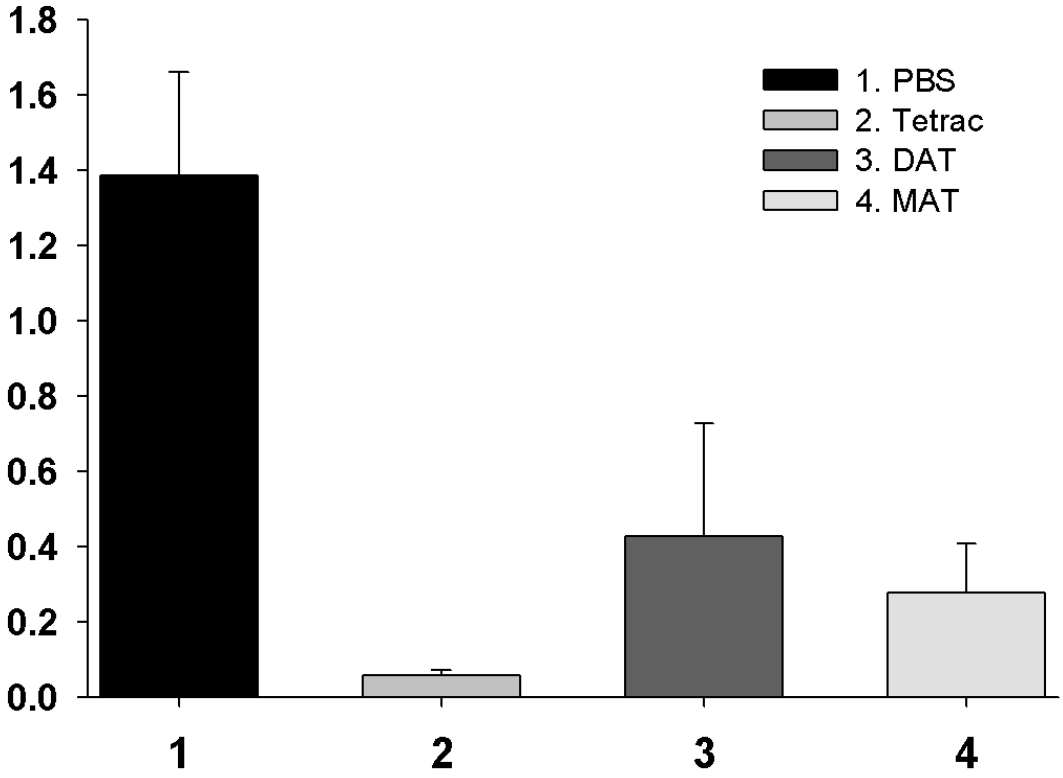


Figure 7. In vivo mouse matrigel model of angiogenesis

Conclusion

Angiogenesis is the formation of new blood vessels from preexisting blood vessels. Design and development of novel angiogenesis inhibitors based on targeting an integrin, has been validated as a target in several tumor types. Integrins are important ligands that play a critical role in the angiogenesis process, particularly blood formation and local release of vascular growth factors, and they are members of a family of cell surface receptors. Tetrac, a deaminated derivative of thyroid hormone, has an interaction site that is located at or near the **RGD** recognition site identified on integrin $\alpha_v\beta_3$'s binding pocket. In order to develop new angiogenesis inhibitors, we

synthesized novel amine derivatives of tetrac (containing mono and di amino group) and tested them for their anti-angiogenesis activity such as binding adhesion study, modeling study as well as *in vivo* mouse matrigel study. Our data showed similar inhibition of glioma cell attachment to fibrinogen. To find the possible binding sites for tetrac, MAT, DAT, we used docking studies to screen the protein surface of the extracellular domain of $\alpha_v\beta_3$, the crystal structure. Inhibition of adhesion of the U87 cells to fibrinogen were tested with increasing concentration of tetrac, MAT and DAT. The cell adhesion to fibrinogen decreased significantly (***$P < 0.01$) in a dose-dependent manner in the presence of compounds until cell binding completely blocked at 1 µM concentration. The inhibition of U87 cells to fibrinogen follows in the order of MAT >DAT > Tetrac at 0.01, 0.03, 0.1, 0.3 and 1 µM concentration. No significance was observed for the binding of tetrac to fibrinogen when compared to cells without any compounds. Auto Dock Vina version 4.0 was employed for the docking of drugs to integrin $\alpha_v\beta_3$ protein and the binding affinities of docked compound were obtained. Mouse matrigel study also showed that tetrac still had higher activity compare to MAT and DAT but MAT showed better activity compare to DAT.

References

1. Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. *New england journal of medicine*, *285*(21), 1182-1186.
2. Velazquez, O. C. (2007). Angiogenesis and vasculogenesis: Inducing the growth of new blood vessels and wound healing by stimulation of bone marrow–derived progenitor cell mobilization and homing. *Journal of vascular surgery*, *45*(6), A39-A47.
3. Rosca, E. V., Koskimaki, J. E., Rivera, C. G., Pandey, N. B., Tamiz, A. P., & Popel, A. S. (2011). Anti-angiogenic peptides for cancer therapeutics. *Current Pharmaceutical Biotechnology*, *12*(8), 1101–1116.
4. Risau, W., Sariola, H.., Zerwes, H. G., Sasse, J., Ekblom, P., Kemler, R., & Doetschman, T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development*, *102*(3), 471-478.
5. Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nature medicine*, *6*(4), 389.
6. Vailhé, B., Vittet, D., & Feige, J. J. (2001). In vitro models of vasculogenesis and angiogenesis. *Laboratory investigation*, *81*(4), 439.
7. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674.
8. Bergers, G., & Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nature reviews cancer*, *3*(6), 401-410.
9. Wicki, A., & Christofori, G. (2008). The angiogenic switch in tumorigenesis. *Tumor Angiogenesis*, 67-88.
10. Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, *86*(3), 353-364.
11. Serini, G., Valdembri, D., & Bussolino, F. (2006). Integrins and angiogenesis: a sticky business. *Experimental cell research*, *312*(5), 651-658.
12. Hynes, R. O. (2002). A reevaluation of integrins as regulators of angiogenesis. *Nature medicine*, *8*(9), 918.
13. Zitzmann, S., Ehemann, V., & Schwab, M. (2002). Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo. *Cancer research*, *62*(18),

5139-5143.

14. Bello, L., et al. (2001). αvβ3 and αvβ5 integrin expression in glioma periphery. *Neurosurgery*, *49*(2), 380-390.
15. Gruber, G. et al. (2005). Correlation between the tumoral expression of β3-integrin and outcome in cervical cancer patients who had undergone radiotherapy. *British journal of cancer*, *92*(1), 41-46.
16. Landen, C. N. et al. (2008). Tumor-selective response to antibody-mediated targeting of αvβ3 integrin in ovarian cancer. *Neoplasia* 10, 1259–126.
17. Hosotani, R. et al. (2002). Expression of integrin αvβ3 in pancreatic carcinoma: relation to MMP-2 activation and lymph node metastasis. *Pancreas* 25, e30–35.
18. McCabe, N. P., De, S., Vasanji, A., Brainard, J. & Byzova, T. V. (2007). Prostate cancer specific integrin αvβ3 modulates bone metastatic growth and tissue remodeling. *Oncogene* 26, 6238–6243.
19. Takayama, S. et al. (2005). The relationship between bone metastasis from human breast cancer and integrin αvβ3 expression. *Anticancer Res.* 25, 79–83.
20. Liapis, H., Flath, A. & Kitazawa, S. (1996). Integrin αVβ3 expression by bone-residing breast cancer metastases. *Diagn. Mol. Pathol.* 5, 127–135.
21. Sloan, E. K. et al. (2006). Tumor-specific expression of αvβ3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Res.* 8, R20.
22. Felding-Habermann, B. et al. (2001). Integrin activation controls metastasis in human breast cancer. *Proc. Natl Acad. Sci.* USA 98, 1853–1858.
23. Albelda, S. M. et al. (1990). Integrin distribution in malignant melanoma: association of the β3 subunit with tumor progression. *Cancer Res.* 50, 6757–6764.
24. Danen, E. H. et al. (1994). Emergence of α5β1 fibronectin- and αvβ3 vitronectin-receptor expression in melanocytic tumour progression. *Histopathology* 24, 249–256.
25. Bates, R. C. et al. (2005). Transcriptional activation of integrin β6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J. Clin. Invest.* 115, 339–347.
26. Hazelbag, S. et al. (2007). Overexpression of the αvβ6 integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival. *J. Pathol.* 212, 316–324.

27. Slack-Davis, J. K., Atkins, K. A., Harrer, C., Hershey, E. D. & Conaway, M. (2009). Vascular cell adhesion molecule-1 is a regulator of ovarian cancer peritoneal metastasis. *Cancer Res.* 69, 1469–1476.
28. Adachi, M. et al. (2000). Significance of integrin α5 gene expression as a prognostic factor in node-negative non-small cell lung cancer. *Clin. Cancer Res*. 6, 96–101.
29. ?
30. Diaz, L. K. et al. (2005). β4 integrin subunit gene expression correlates with tumor size and nuclear grade in early breast cancer. *Mod. Pathol.* 18, 1165–1175.
31. Friedrichs, K. et al. (1995). High expression level of α6 integrin in human breast carcinoma is correlated with reduced survival. *Cancer Res.* 55, 901–906.
32. Avraamides, C. J.; Garmy-Susini, B.; Varner, J. A. Nat. Rev. Cancer 2008, 8, 604–617.
33. Serini, G.; Valdembri, D.; Bussolino, F. Exp. Cell Res. 2006, 312, 651–8.
34. Hynes, R. O. Nat. Med. 2002, 8, 918–921.
35. Pierschbacher, M. D.; Ruoslahti, E. Nature 1984, 309, 30–33.
36. Jaracz, S.; Chen, J.; Kuznetsova, L. V; Ojima, I. Bioorg. Med. Chem. 2005, 13, 5043–5054.
37. Zitzmann, S.; Ehemann, V.; Schwab, M. Cancer Res. 2002, 62, 5139–43.
38. Bogdanowich- Knipp, S. J., Chakrabarti, S., Siahaan, T. J., Williams, T. D., & Dillman, R. K. (1999). Solution stability of linear vs. cyclic RGD peptides. Chemical Biology & Drug Design, 53(5), 530-541.
39. Li, Z. J., & Cho, C. H. (2012). Peptides as targeting probes against tumor vasculature for diagnosis and drug delivery. Journal of translational medicine, 10(1), S1.
40. Luidens, M. K., Mousa, S. A., Davis, F. B., Lin, H. Y., & Davis, P. J. (2010). Thyroid hormone and angiogenesis. *Vascular pharmacology*, *52*(3), 142-145.
41. Bridoux, A., Cui, H., Dyskin, E., Yalcin, M., & Mousa, S. A. (2009). Semisynthesis and pharmacological activities of Tetrac analogs: angiogenesis modulators. *Bioorganic & medicinal chemistry letters*, *19*(12), 3259-3263.
42. Yalcin, et al. (2010). Tetraiodothyroacetic acid (tetrac) and nanoparticulate tetrac arrest growth of medullary carcinoma of the thyroid. *The Journal of Clinical Endocrinology & Metabolism*, *95*(4), 1972-1980.

43. Mousa, et al. (2008). Tetraiodothyroacetic acid, a small molecule integrin ligand, blocks angiogenesis induced by vascular endothelial growth factor and basic fibroblast growth factor. *Angiogenesis*, *11*(2), 183-190.
44. Mousa, et al. (2012). Tetraiodothyroacetic acid and its nanoformulation inhibit thyroid hormone stimulation of non-small cell lung cancer cells in vitro and its growth in xenografts. *Lung Cancer*, *76*(1), 39-45.
45. Yoshida, T., Gong, J., Xu, Z., Wei, Y., & Duh, E. J. (2012). Inhibition of pathological retinal angiogenesis by the integrin αvβ3 antagonist tetraiodothyroacetic acid (tetrac). *Experimental eye research*, *94*(1), 41-48.
46. Yalcin, et al. (2010). Tetraiodothyroacetic acid (tetrac) and nanoparticulate tetrac arrest growth of medullary carcinoma of the thyroid. *The Journal of Clinical Endocrinology & Metabolism*, *95*(4), 1972-1980.
47. Bharali, D. J., Yalcin, M., Davis, P. J., & Mousa, S. A. (2013). Tetraiodothyroacetic acid-conjugated PLGA nanoparticles: a nanomedicine approach to treat drug-resistant breast cancer. *Nanomedicine*, *8*(12), 1943-1954.
48. Yalcin et al. (2009). Tetraidothyroacetic acid (tetrac) and tetrac nanoparticles inhibit growth of human renal cell carcinoma xenografts. *Anticancer research*, *29*(10), 3825-3831.
49. Srinivasan, M., Rajabi, M., & Mousa, S. A. (2015). Multifunctional nanomaterials and their applications in drug delivery and cancer therapy. *Nanomaterials*, *5*(4), 1690-1703.
50. Srinivasan, M., Rajabi, M., & Mousa, S. A. (2016). Nanobiomaterials in Cancer Therapy. In *Nanobiomaterials in Cancer Therapy: Applications of Nanobiomaterials* (Vol. 7, p. 57). Elsevier Amsterdam.
51. Rajabi, M., Srinivasan, M., & Mousa, S. A. (2016). Nanobiomaterials in Drug Delivery. In *Nanobiomaterials in Drug Delivery: Applications of Nanobiomaterials* (Vol. 9, p. 1). Elsevier Amsterdam.
52. Rajabi, M., Srinivasan, M., & Mousa, S. A. (2016). Nanobiomaterials in Drug Delivery. In *Nanobiomaterials in Drug Delivery: Applications of Nanobiomaterials* (Vol. 9, p. 1). Elsevier Amsterdam.
53. Rajabi, M., Sudha, T., Darwish, N. H., Davis, P. J., & Mousa, S. A. (2016). Synthesis of MR-49, a deiodinated analog of tetraiodothyroacetic acid (tetrac), as a novel pro-

angiogenesis modulator. *Bioorganic & Medicinal Chemistry Letters*, *26*(16), 4112-4116.

54. Lin, H.-Y.; Chin, Y.-T.; Nana, A. W.; Shih, Y.-J.; Lai, H.-Y.; Tang, H.-Y.; Leinung, M.; Mousa, S. A.; Davis, P. J. (2016) *Steroids*, *15*, 943–958.
55. Wu, S. Y., Green, W. L., Huang, W. S., Hays, M. T., & Chopra, I. J. (2005). Alternate pathways of thyroid hormone metabolism. *Thyroid*, *15*(8), 943-958.
56. Still, W. C., Kahn, M., & Mitra, A. (1978). Rapid chromatographic technique for preparative separations with moderate resolution. *The Journal of Organic Chemistry*, *43*(14), 2923-2925.
57. Bridoux, A.; Khan, R. A.; Chen, C.; Chevé, G.; Cui, H.; Dyskin, E.; Yasri, A.; Mousa, S. (2011) *A. J. Enzyme Inhib. Med. Chem.*, 26, 871–88
58. Huang, A. Y.-T.; Tsai, C.-H.; Chen, H.-Y.; Chen, H.-T.; Lu, C.-Y.; Lin, Y.-T.; Kao, C.-L. (2013). *Chem. Commun. (Camb).*, 49, 5784–6.
59. Moloney, M. G. (2002). Excitatory amino acids. *Natural product reports*, *19*(5), 597-616.
60. Hao, J., Reinhard, M., Henry, S. S., Seest, E. P., Belvo, M. D., & Monn, J. A. (2012). Simple conversion of fully protected amino acids to zwitterions. *Tetrahedron Letters*, *53*(12), 1433-1434.
61. Davis, P. J., Davis, F. B., Mousa, S. A., Glinksy, G. V., & Hercbergs, A. (2015). *U.S. Patent No. 9,220,788*. Washington, DC: U.S. Patent and Trademark Office.