Characterization of Small Molecules Inhibiting Pro-angiogenic Activity of the Zinc Finger Transcription Factor Vezf1

He Ming[1], Qianyi Yang[4], Allison Norvil[1], David Sherris[3] and Humaira Gowher[1, 2]*

[1]Department of Biochemistry, [2]Purdue University Center for Cancer Research,

Purdue University, West Lafayette, Indiana 47907

[4]Present address: Department of Anesthesiology, Washington University School of Medicine, 660 S Euclid Ave, St. Louis, MO 63110

[3]Present address: GenAdam Therapeutics, Inc., 37 Neillian Crescent, Jamaica Plain, MA 02130

*Corresponding author: hgowher@purdue.edu; 3018202794

Running title: Small molecule inhibition of Vezf1 activity in endothelial cells.

Keywords: Vezf1; angiogenesis; vascular biology; endothelial cells; MSS31, tube formation, small molecule inhibitors computational modeling.

Abstract

Discovery of inhibitors for endothelial-related transcription factors can contribute to the development of angiogenic therapies that treat diseases ranging from cardiovascular to cancer. The role of transcription factor Vezf1 in vascular development and regulation of angiogenesis has been defined by several earlier studies. Through construction of a computational model for Vezf1, work here has identified a novel small molecule drug capable of inhibiting Vezf1 from binding to its cognate DNA binding site. Using structure-based design and virtual screening of the NCI Diversity Compound Library, 12 shortlisted compounds were tested for their ability to interfere with the binding of Vezf1 to DNA using electrophoretic gel mobility shift assays. We identified one compound, T4, which has an IC50 of 20uM. Using murine endothelial cells, MSS31, we tested the effect of T4 on endothelial cell viability and angiogenesis by using tube formation assay. Our data show that addition of T4 in cell culture medium does not affect cell viability at concentrations lower or equal to its IC 50 but strongly inhibits the network formation by MSS31 in the tube formation assays. Given its potential efficacy, this inhibitor has significant therapeutic potential in several human diseases raging from wound healing to cancer.

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

Introduction

Vezf1 (Vascular Endothelial Zinc Finger 1), previously named Bgp1, was discovered as a protein binding to a poly (dG) sequence present in the neighborhood of chicken β-globin promoter (1,2). Vezf1 belongs to the family of Kruppel-like zinc finger protein that contains six C2H2 class zinc finger motifs (~99% identical between homologues. It is about 65kd protein and recognizes long strings of poly (dG).poly (dC) ('G-strings'). Biochemical studies revealed that the minimal binding site of Vezf1 is a $(dG)^7$ string, but it also binds to a bipartite poly G string containing a $(dG)^6$ and $(dG)^4$ separated by three to four nucleotides present in the upstream hypersensitive site 5'HS4 of chicken β- globin locus (3). Interestingly, poly G tracts are highly prevalent in CpG islands in mammalian genomes resulting in a number of putative binding sites in these regions. Similar to some other zinc finger proteins such as CTCF, Vezf1 is conserved on among vertebrates and the proteins are near identical between mouse and human (4,5).

Vezf1 is ubiquitously expressed but it is highly expressed in vascular endothelium during early development. Targeted inactivation if Vezf1 gene in mice causes embryonic lethality and it acts in a dosage dependent fashion to regulate the development of blood and lymphatic vascular system (6,7). Embryonic stem cells (ESCs) derived from $Vezf1^{-/-}$ embryos differentiated into embryoid bodies were shown to have defect sprouting angiogenesis (8). However these studies also showed that loss of Vezf1 had no significant effect on the expression of pioneer factors that regulate vasculogenesis, indicating a complex mechanism. Other studies have shown the function of Vezf1 in regulation of adult angiogenesis (9,10). Using primary human endothelial cells (BVECs), Vezf1 was shown to be functionally linked with Rho B which is a small GTPase known to be involved in angiogenesis during post-natal retinal development (11,12). Our recent genome-wide gene expression analysis of the $Vezf1^{-/-}$ ESCs showed a significant high expression of the antiangiogenic factor Cited2/Mrg1 (13). Using an in vitro endothelial cell (EC) differentiation and tube formation as model system for angiogenesis, our data showed in the some of the defects could be rescued by reducing Cited2 expression in $Vezf1^{-/-}$ ESC derived ECs (unpublished data). To understand the mechanism, of Vezf1, our previous studies showed a major loss of DNA methylation genome-wide with a concomitant reduction in the expression of a major DNA methyltransferase Dnmt3b in $Vezf1^{-/-}$ ESCs (14). We further showed that Vezf1 binding sites overlap with the RNA polymerase 2 pausing sites genome-wide and interacts with chromatin modifying proteins (13). However the details of Vezf1 mechanism and how it impacts gene expression in early development or in somatic cells in not known. However several studies have demonstrated its vital role in both embryonic and postnatal angiogenesis (5).

The development vascular system is the most critical and earliest step in development and one of the critical requirements for tumor progression and metastasis (15,16). Several studies have led to the identification of many transcription factors that regulate angiogenesis but most of them are not

Peer-reviewed version available at *Molecules* **2018**, *23*, 1615; doi:10.3390/molecules23071615

endothelial specific thus making it difficult to use them as targets for anti-cancer drug development (17,18) however, Vezf1, is unique among endothelial factors since during development its function is restricted to vascular system thus making it an attractive target for cancer therapeutics (6). The factors that drive these developmental processes include the ligand-receptor signaling players VEGF/VEGFR, angiopoietin/Tie2 and the Ephrin/Eph receptor system. Currently approved angiogenesis inhibitors target the VEGF pathway by direct inhibition of VEGF or VEGF receptors and few explore alternatives to this direct inhibition (19,20). New endothelial-related transcription factors can contribute to the development of antiangiogenic therapies that treat diseases ranging from cardiovascular disease to cancer. The need for new cancer therapeutics and the necessity to understand the complex mechanisms involved in regulation of cell-type specific genes has increased the demand for comprehensive characterization of small molecule inhibitors against factors such as Vezf1 which can potentially work in a restricted fashion and block angiogenesis and hyper-vascularization in diseased state.

In the present study we used recombinant Vezf1 protein to characterize 12 compounds that could potentially inhibit its binding to DNA. We found T4 to block Vezf1-DNA binding at low uM concentration. We tested the in vivo toxicity and activity of T4 by using MSS31 endothelial cells. We show a specific inhibition of tube formation by MSS31 cells which are viable when treated with a dose equal to IC50 of T4. We therefore report an a effective compound that can potentially block angiogenesis without affecting cell viability.

RESULTS

Determination of DNA binding constant Vezf1 to its specific DNA sequence.

In order to perform the biochemical testing of the potential small molecule inhibitors, we first determined the DNA binding constant of the recombinant His-Vezf1. The murine His-tagged Vezf1 was purified using the bacterial overexpression system (Figure 1A). Gel mobility shift analysis was used to analyze the interactions of His- Vezf1 recombinant protein with 32P-labelled oligonucleotides containing Vezf1 binding sites characterized at the chicken beta-globin insulator element. Binding assays were carried by incubating 25 nM of radiolabeled oligonucleotides with two-fold increasing concentration of protein ranging from 50-1000 nM in the binding buffer. Protein bound DNA runs as a slower species on the gel (Figure 1B). The band intensities are used to determine the ratio of bound to unbound nucleic acid on the gel which reflects the fraction of free and bound probe molecules as the binding reaction enters the gel. The data were fitted to the following expression which directly follows from the definition of a bimolecular binding equilibrium and was used to determine the binding constant (K_{dis}) of Vezf1.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

$$\upsilon_1 = \frac{D_{tot} + P_{tot} + K_{dis} - \sqrt{(D_{tot} + P_{tot} + K_{dis})^2 - 4 \times D_{tot} \times P_{tot}}}{2 \times D_{tot}}$$

V_1 is the fractional degree of saturation of the available protein binding sites on the DNA at increasing protein concentrations. The dissociation constant of Vezf1 as shown in Figure 1C was approximately 640 nM.

FIGURES

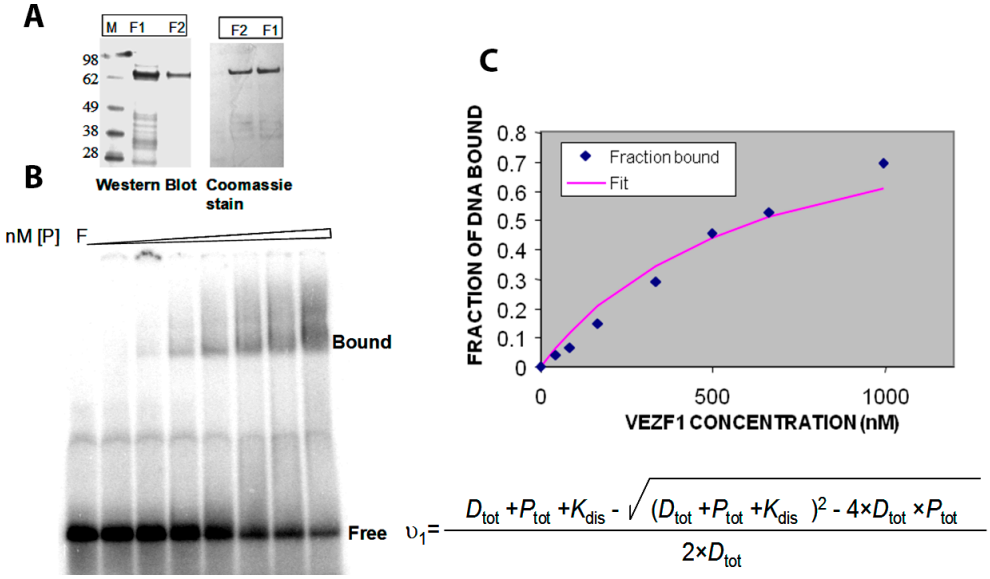


Figure 1: A) His tagged Vezf1 was purified with affinity chromatography using Ni-NTA column. F1 and F2 represent the eluted fractions 1 and 2 which are on SDS PAGE stained with Commassie blue. The integrity of the recombinant protein was tested by Western blot probed with anti-His antibody. B) EMSA (electrophoretic mobility shift assay) was performed to determine the DNA binding constant of Vezf1. 25 nM of radiolabeled DNA were incubated with increasing amount of purified Vezf1 protein 50-1000nM). C) The band intensities of the free and bound fractions were measured using Image Quant L software. The data were fitted into an equation of binding equilibrium to determine binding constant of 640 nM.

Effect of small molecule inhibitors on DNA binding property of Vezf1

Next, we tested the effect of candidate inhibitors (Table 1) on the DNA binding affinity of Vezf1. Vezf1 protein at 640 nM concentration was incubated with 500 uM of potential inhibitors for 10 min. All the 12 compounds tested are soluble in water and/or DMSO. 25 nM labeled DNA was added to the reaction and binding assays were done as described above (Figure. 2A). The bound and the free DNA fractions were quantified and plotted (Figure 2B). Various compounds had varying effect on the DNA binding ability of Vezf1. 3 of the 12 tested compounds, A, B and C, had the greatest degree of

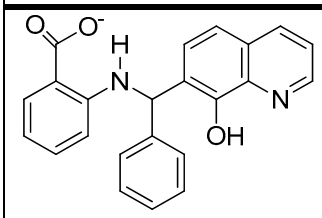
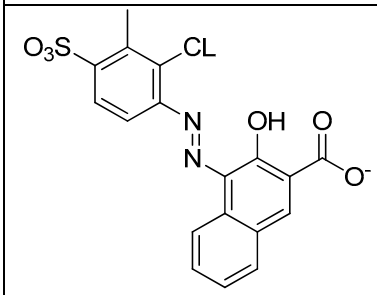
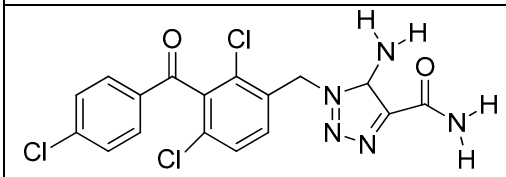
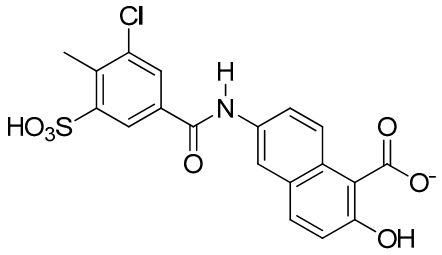
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

potency resulting in a near complete loss of DNA binding by Vezf1. The percent bound DNA is also shown in Table 1.

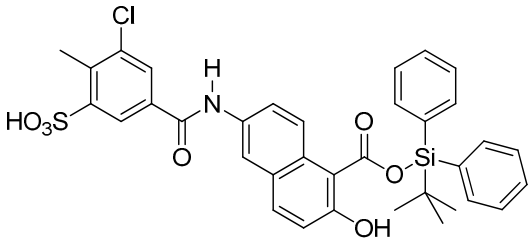
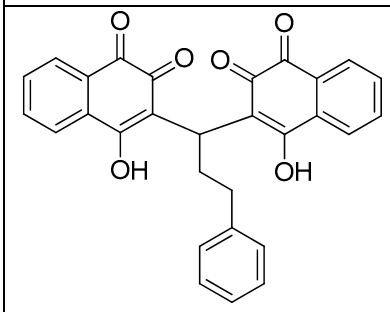
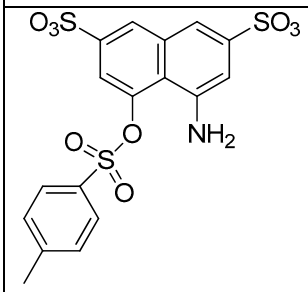
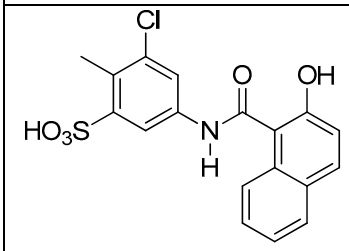
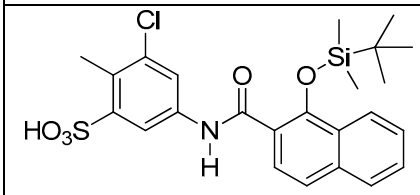
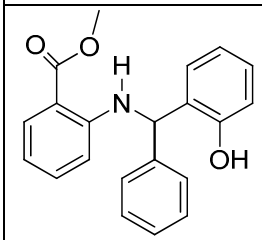
Next we determined the minimum concentration at which these 3 compounds were effective, and determine the IC50. DNA binding assays were done by pre-incubating 1 uM Vezf1 with varying concentrations of the inhibitors and test the ability of Vezf1 to bind DNA using the gel shift assays. Out of the 3 small molecule inhibitors, **T4** (503-1-83) was most potent. It was able to block DNA binding by Vezf1 effectively at a concentration of 20 uM. **T6** (503-1-71) and NSC1012 inhibited DNA binding by Vezf1 at concentrations of 100 uM and 500 uM respectively (Figure 2C).

Table 1. Summary of the compounds used and their activity

Compound Structure	Compound Number	% DNA bound in EMSA (500 uM)	Activity Concentration
	NSC1012 (**1**)	7.38%	500 uM
	NSC16087 (**2**)	22.56%	500 uM
	NSC609974 (**3**)	38.45%	500 uM
	**T4**	5.94%	20 uM

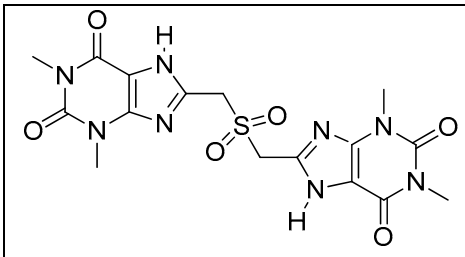
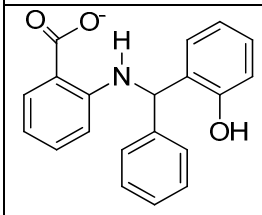
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at *Molecules* **2018**, *23*, 1615; doi:10.3390/molecules23071615

	**T6**	5.92%	100 uM
	CID 272651 (NSC117274)	27.13%	NA
	CID 23270	29.37%	NA
	**T3**	30.81%	NA
	**T5**	36.81%	NA
	**T2**	38.23%	

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

	CID 267103	38.95%	
	**T1**	43.58%	

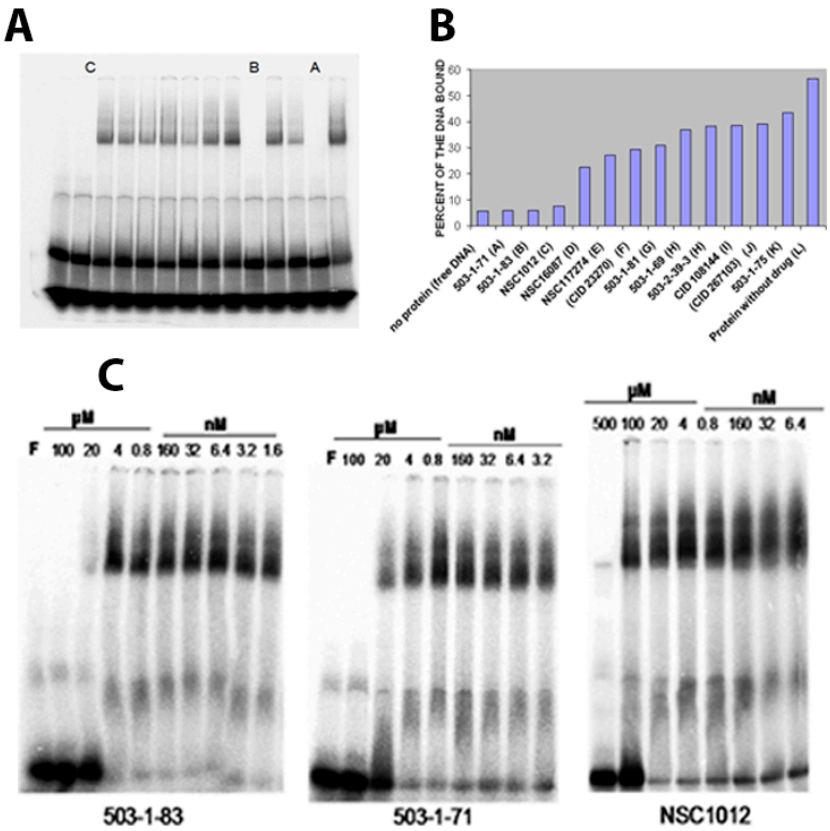


Figure 2: A) EMSA showing the effect of candidate inhibitors on the DNA binding affinity of Vezf1. The protein was pre incubated 500 mM of potential inhibitors for 10 min before adding the DNA to the binding reaction. Compounds noted as A, B and C . Presence of compounds have varying effect on the DNA binding ability of VEZF1. 3 of the 12 tested compounds, A, B and C, severely affected DNA binding of the recombinant Vezf1. B) The bound and free reactions were quantified and plotted as percent bound. A, B and C compounds were identified as 501-1-71, 503-1-83 and NSC1012

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

respectively. C) To determine the minimum concentration at which these 3 drugs were effective, DNA binding assays were done by pre-incubating 640 nM Vezf1 with varying concentrations of the 3 selected inhibitors, followed by DNA binding assay. As shown, 503-1-83 was most effective in blocking DNA binding by Vezf1 at 20 uM, whereas 503-1-71 showed similar affect at about 100uM and NSC1012 at 500uM.

Effect of small molecule inhibitors on cell viability

Following the biochemical tests, we wanted to to test the ability of these compounds to inhibit Vezf1 activity in cells. However, we first tested the cellular toxicity of some selected compounds. We tested four candidates: T4: most potent based on IC50, followed by T6 and T5. We also tested T2 as a control which has no effect in vitro on Vezf1 DNA binding but it has a similar structure to T4 (Figure 3A). Due its critical role in endothelial development and angiogenesis, we used MSS31, mouse endothelial cell line, for these studies. MSS31 cells were treated with increasing concentration of the four inhibitors for 24 hours. Cells were collected and the live cell population was counted by Bio-Rad cell counter using Trypan Blue staining. As shown in Figure 3B, exposure to T2 and T6 causes cell death at very low concentration. T4 is less toxic and can be tolerated until 50 um. These data show that T4 can be tolerated by cells at concentrations close to its estimated IC50.

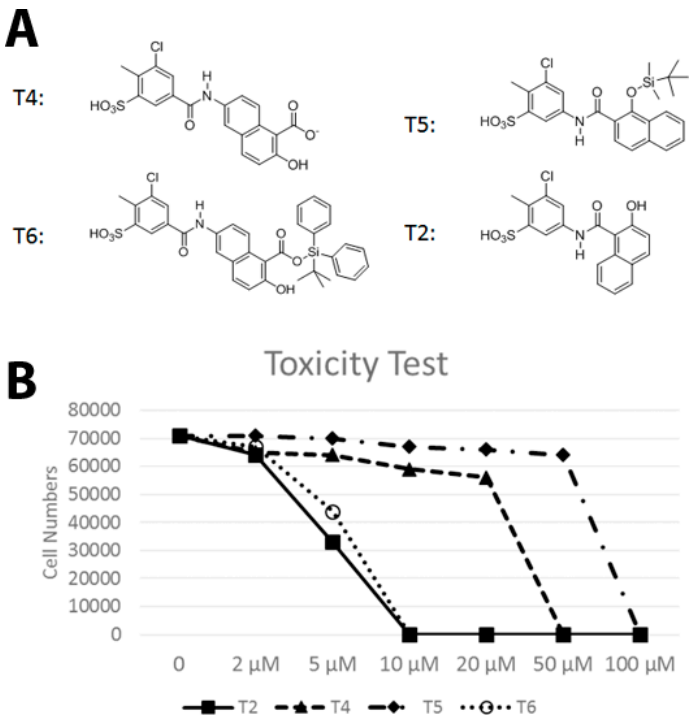


Figure 3: A) EMSA showing the effect of candidate inhibitors on the DNA binding affinity of Vezf1. The protein was pre incubated 500 mM of potential inhibitors for 10 min before adding the DNA to the

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 May 2018 doi:10.20944/preprints201805.0209.v1

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

binding reaction. Presence of compounds have varying effect on the DNA binding ability of VEZF1. 3 of the 12 tested compounds, A, B and C, severely affected DNA binding of the recombinant Vezf1. B) The bound and free reactions were quantified and plotted as percent bound. A, B and C compounds were identified as 501-1-71, 503-1-83 and NSC1012 respectively. C) To determine the minimum concentration at which these 3 drugs were effective, DNA binding assays were done by pre-incubating 640 nM Vezf1 with varying concentrations of the 3 selected inhibitors, followed by DNA binding assay. As shown, 503-1-83 was most effective in blocking DNA binding by Vezf1 at 20 uM, whereas 503-1-71 showed similar affect at about 100uM and NSC1012 at 500uM.

T4 treated MSS31 cells are incapable of tube formation in Matrigel

Vezf1 has a critical function in differentiation of endothelial cells and angiogenesis. Angiogenesis can be modeled in tissue culture by plating MSS31 endothelial cells in VEGF containing Matrigel. The cells arrange into distinct tube like structures within 6-10 hours. To determine the ability of the small molecule inhibitors to disrupt this biological activity of Vezf1, we tested the effect of T4 and T5 on the tube formation by endothelial cells MSS31. We exposed MSS31 cells to T4 at 20uM, a concentration close to its estimated IC50 value and T5 at 75 um, given at 100 um it is lethal for 24 hours. Treated and untreated MSS31 cells were plated in Matrigel. Within 6-10 hours the untreated cells organized into networks as shown by phase contrast microscopy (Figure 4A). The cells treated with T5 were not at all affected and formed networks similar to untreated cells, however the network formation by cells treated with T4 were strongly inhibited. The tube length was measured which shows a clear absence of tube formation by T4 treated MSS31 cells (Figure 4B)

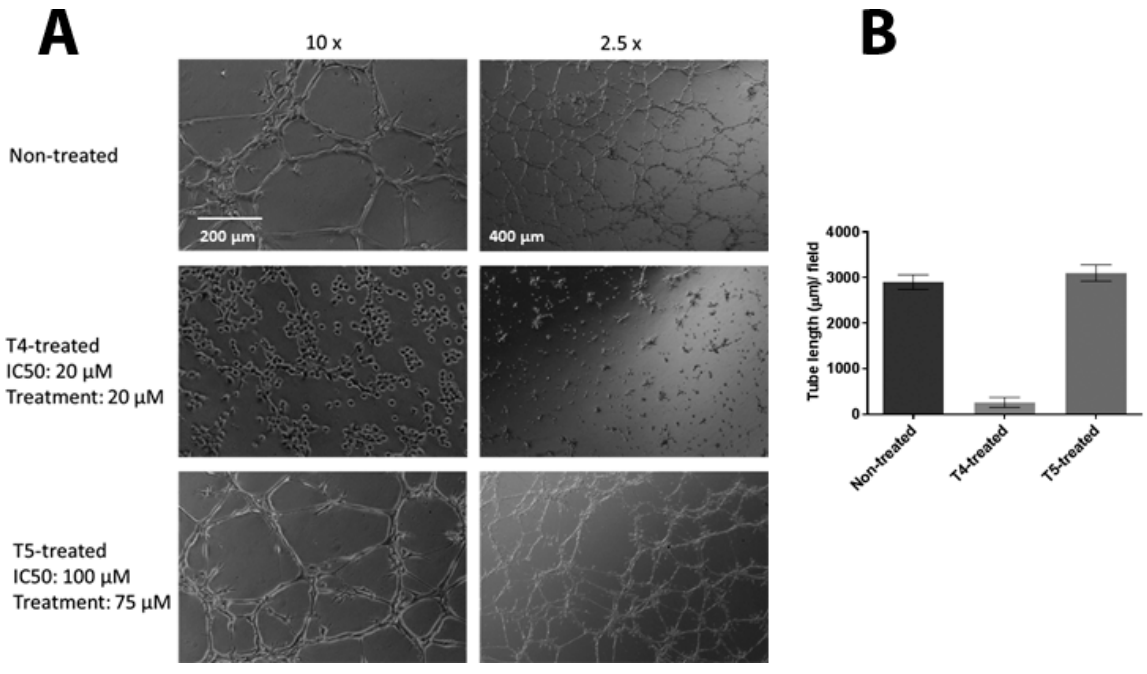


Figure 4: Effect of Vezf1 inhibitors on its biological activity. A) MSS31 cells were treated with 20 μM compound T4 or 75 μM compound T5 for 24 hours. Cells were placed on Matrigel, and tube formation was evaluated after 18 hours by bright field microscopy. T4 treatment clearly prevents tube formation in MSS31 cells compare to non-treated and T5-treated cells. 10X and 2.5X show two magnifications of cells in Matrigel. B) Tube length in A was measured using Image Quant L software and plotted. T4 treated cells show no tube formation whereas T5 treated cells, the tube length is similar to that of the untreated cells.

These data strongly support T4 to be inhibitor of Vezf1 activity in endothelial cells and has ability to block angiogenesis at non-toxic concentrations. The endothelial cells that can be further tested in vivo for its ability to block angiogenesis. This can be performed by using the inhibitor in wound healing assays and as a potential therapeutic to control angiogenesis in growing tumors in model organisms.

DISCUSSION

Development of a proper vascular system is indispensable for embryonic development during which an accurate spatial and temporal control of gene expression is required in endothelial cells committed to the formation of vasculature. Angiogenesis is regulated by the activity of several proteins including Vezf1. Several factors, like VEGF, VEGFR, and ET-1 have been studied and the mechanistic details of their activity in angiogenesis have been worked out (15). The discovery of Vezf1 as an endothelial factor and its role in angiogenesis is relatively new. Genetic knockout studies revealed the role of Vezf1 in vascularization, however the mechanism of its regulation is not fully understood (6). Our previous studies have characterized the activity of Vezf1 at chicken b-globin insulator. Insulators are chromatin elements that block the non-specific activity of enhancers by disallowing their interaction with unassigned promoters (21). However the mechanism by which Vezf1 regulates gene expression and angiogenesis during development and disease is unknown. Through construction of a new computational model for Vezf1, work here has identified a potential small molecule binding site (occupied by DNA in the X-ray structure used to construct the model) for use in creating novel small molecule drugs capable of inhibiting Vezf1 from binding to its cognate DNA binding site. Using AlleGrow and docking tools of FLO to design new compounds, a novel compound was discovered which inhibits binding of DNA at 20uM concentration. Of particular note, out of 12 designed compounds, identification of such a small molecule inhibitor may be considered a significant achievement.

Discovery of a small molecular inhibitor against Vezf1 will not only have therapeutic use, but can also be employed to understand its mechanism in development and disease. Tumor cells treated with these inhibitors can be tested for proliferation, migration and tube formation. Further this inhibitor can

be used to study wound healing in animal models. This new lead compound is also a candidate for the determination of an X-ray crystal structure of the zinc finger/ligand complex. It is also ideal as a starting point for new rounds of design, synthesis and biological evaluation to find medicinal agents interfering with zinc finger/DNA interactions, a new mechanism of action for development of agents against cancer.

MATERIALS AND METHODS

Computational modelling of Vezf1 structure, evaluation of potential binding sites and design of small molecule inhibitors

Computational modeling and design of small molecules was done at Vasculomedics Inc. Briefly, a model of the zinc finger, Vezf1 that would compare favorably to the experimentally determined structures of other zinc fingers was determined. Using high resolution DNA bound structure of Zinc Finger 268 and 1AAY, the structure was further refined using Monte Carlo searching. VasculoMedics, Inc. had previously tested few lead compounds that inhibit Vezf1 DNA binding using previously designed model of Vezf1. Docking tools of FLO (McDock+) were used to dock these known ligands (Supplementary figure 1, 2). New compounds were designed such that they incorporate only minimal changes from the lead compounds thus using docking experiments of lead compounds as a guide. The de novo program AlleGrow was used to discover novel side chains to replace those of the leads. Each designed compound was docked and evaluated in the model. Those structures were selected which formed the most favorable interactions and had good energy profiles. After evaluation of several lead compounds for complexity in synthesis, six compounds were selected and synthesized at MedChem Partners. Next, a major virtual screening program was performed in order to find compounds at the NCI Diversity Compound Library.

Virtual Screening

We used major virtual screening program to find compounds that could be obtained from the NCI Diversity Compound Library. The first step for virtual screening was to identify the docking protocol, which gave the best result when docking compounds of known activity. Therefore, a number of different docking algorithms were tested by docking all compounds. The methods tested included:

1) **SDock+.** Sdock+ is a very fast docking algorithm that uses a novel method for generating vast numbers of conformers in the target-binding site. The best scoring conformers are energy minimized in the binding site.

2) **McDock+** McDock+ uses a Monte Carlo algorithm for generating new conformers. Conformers generated by McDock+ are optimized with energy minimization and the FLO scoring function. The calculations are much more time consuming than Sdock+, however, often the Mcdock+ results are more reliable.

Peer-reviewed version available at Molecules 2018, 23, 1615; doi:10.3390/molecules23071615

3) **SDock+ followed by McDock+** Conformers found by SDock+ served as a starting point for McDock+.

Upon finding positive compounds selected from virtual screening in the NCI database, each compound was evaluated in the model (Supplementary figure 3). Those forming the most favorable interactions were selected, for synthesis by MedPharma Partners. All compound synthesized are shown in Table 1.

Cloning and Purification of recombinant Vezf1

The Vezf1 gene was cloned to encode an N-terminal His6-tag fusion protein into pQE10 (Qiagen). DH5α, BL21(DE3, pLysS) or XLBlue (MRF, T7) E. coli cells, transformed with pQE10 Vezf1, were grown at 32°C in 500 ml of LB medium containing 75 mg/ml ampicillin. Protein expression was induced at a cell density of 0.3 A600 nm by the addition of 1 mM IPTG and the cells were grown for an additional two hours at 30°C. All purification steps were carried out at 4°C. The cells were harvested by centrifugation and washed with STE buffer (10 m Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 M NaCl). The cell pellet was suspended in buffer A (20 mM KPi (pH 7.5), 1 mM EDTA, 0.1 mM DTT, 0.5 M NaCl, 10% (v/v) glycerol, 20 mM imidazole) and the cells were disrupted by sonication. Since the Vezf1 protein turned out to be susceptible to proteolysis, the purification was carried out in the presence of Protease Inhibitor Cocktail (Roche) in the sonication buffer. Cell debris was removed by centrifugation (60 minutes, 13,000 g). The supernatant was applied onto a Ni-NTA (Qiagen) column (1 ml gel bed) equilibrated with buffer A. After washing with 150 ml of buffer A, the His- Vezf1 was eluted with 5 ml of elution buffer (20 mM KPi (pH 7.5), 1 mM EDTA, 0.1 mM DTT, 0.5 M NaCl, 10% (v/v) glycerol, 200 mM imidazole). The eluate was dialyzed overnight against storage buffer (20 mM Hepes (pH 7.5), 40 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 20% glycerol) aliquoted and stored at -80°C. The Vezf1 concentration was estimated from Coomassie blue-stained SDS-PAGE gels using protein standards of known concentration and Western blots were carried out using an anti-His6-tag antibody (abcam), according to the instructions of the supplier.

DNA binding assays

50 pmoles of oligonucleotide were end-labeled by polynucleotide kinase in presence of γ [32P] ATP. Purified recombinant Vezf1 was allowed to bind to the oligonucleotide containing the Vezf1 binding site in HS4-FPIII. The reaction was carried out in 15ul volume in binding buffer (20mM Hepes 7.5, 10mM MgCl2, 50mM NaCl, 1mM EDTA, 5ng/ul poly dA.dT) and incubated for 10 min at room temperature followed by the addition of 5% Ficoll.

The bound and the free DNA was separated on 5% acrylamide gel run in .5XTBE buffer for 2 hrs at 150V. The gel was dried and imaged by PhosphoImager. The band densities were quantified by the ImageQuant TL software. The top strand sequence for the duplex used for gel mobility shift analyses

is 5' AGGCGCGCCCCGGTCCGGCGCTCCCCCCGCATCCCCGAGCCGGGGCGCGCCT 3'. The relative band intensities were used to fit the data to an equation representing bimolecular binding equilibrium using Microsoft excel and the binding constant was determined.

Small molecule inhibitors

The small molecule synthesized by MedPharma Partners were suspended in DMSO at 10mg/ml concentration. Further dilutions for use in the gel shift assays and cell culture were done in water. The diluted samples were stored in -20C whereas the stock in DMSO was stored in 4C.

Tube formation assays:

MSS31 mouse endothelial cells were collected by trypsinization and counted using Bio-Rad Cell Counter. The tube formation or sprouting assay was performed by plating $2x10^5$ cells on a 24-well plate coated with VEGF supplemented Matrigel (BD Biosciences)according to the manufacturers protocol [39, 40]. The cells were incubated at 37 degrees for 3-18 hours. Sprouting was scored using images from phase contrast microscopy [31]. The length of the tubes was measured by Image J software. For inhibitor treatment, MSS31 cells were treated for 24hrs with compounds, after which cells were trypisinized and plated on Matrigel for tube formation assays.

Contributions: HG and DS designed the experiments, MH, QY and AN performed the experiments and HG wrote the manuscript.

Funding: This work was supported by Scientist Development Award from American Heart Association to HG, ACS institutional grant from PUCCR supported by P30 CA0231681 and R43 GM093385-01 to DS.

The authors declare no conflict of interest

References

1. Lewis, C. D., Clark, S. P., Felsenfeld, G., and Gould, H. (1988) An erythrocyte-specific protein that binds to the poly(dG) region of the chicken beta-globin gene promoter. *Genes Dev* **2**, 863-873
2. Clark, S. P., Lewis, C. D., and Felsenfeld, G. (1990) Properties of BGP1, a poly(dG)-binding protein from chicken erythrocytes. *Nucleic Acids Res* **18**, 5119-5126
3. Dickson, J., Gowher, H., Strogantsev, R., Gaszner, M., Hair, A., Felsenfeld, G., and West, A. G. (2010) VEZF1 elements mediate protection from DNA methylation. *PLoS genetics* **6**, e1000804
4. Weth, O., and Renkawitz, R. (2011) CTCF function is modulated by neighboring DNA binding factors. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **89**, 459-468
5. Bruderer, M., Alini, M., and Stoddart, M. J. (2013) Role of HOXA9 and VEZF1 in endothelial biology. *Journal of vascular research* **50**, 265-278

6. Kuhnert, F., Campagnolo, L., Xiong, J. W., Lemons, D., Fitch, M. J., Zou, Z., Kiosses, W. B., Gardner, H., and Stuhlmann, H. (2005) Dosage-dependent requirement for mouse Vezf1 in vascular system development. *Developmental biology* **283**, 140-156
7. Aitsebaomo, J., Kingsley-Kallesen, M. L., Wu, Y., Quertermous, T., and Patterson, C. (2001) Vezf1/DB1 is an endothelial cell-specific transcription factor that regulates expression of the endothelin-1 promoter. *The Journal of biological chemistry* **276**, 39197-39205
8. Zou, Z., Ocaya, P. A., Sun, H., Kuhnert, F., and Stuhlmann, H. (2010) Targeted Vezf1-null mutation impairs vascular structure formation during embryonic stem cell differentiation. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 1378-1388
9. Miyashita, H., and Sato, Y. (2005) Metallothionein 1 is a downstream target of vascular endothelial zinc finger 1 (VEZF1) in endothelial cells and participates in the regulation of angiogenesis. *Endothelium : journal of endothelial cell research* **12**, 163-170
10. Miyashita, H., Kanemura, M., Yamazaki, T., Abe, M., and Sato, Y. (2004) Vascular endothelial zinc finger 1 is involved in the regulation of angiogenesis: possible contribution of stathmin/OP18 as a downstream target gene. *Arteriosclerosis, thrombosis, and vascular biology* **24**, 878-884
11. Gerald, D., Adini, I., Shechter, S., Perruzzi, C., Varnau, J., Hopkins, B., Kazerounian, S., Kurschat, P., Blachon, S., Khedkar, S., Bagchi, M., Sherris, D., Prendergast, G. C., Klagsbrun, M., Stuhlmann, H., Rigby, A. C., Nagy, J. A., and Benjamin, L. E. (2013) RhoB controls coordination of adult angiogenesis and lymphangiogenesis following injury by regulating VEZF1-mediated transcription. *Nature communications* **4**, 2824
12. Aitsebaomo, J., Wennerberg, K., Der, C. J., Zhang, C., Kedar, V., Moser, M., Kingsley-Kallesen, M. L., Zeng, G. Q., and Patterson, C. (2004) p68RacGAP is a novel GTPase-activating protein that interacts with vascular endothelial zinc finger-1 and modulates endothelial cell capillary formation. *The Journal of biological chemistry* **279**, 17963-17972
13. Gowher, H., Brick, K., Camerini-Otero, R. D., and Felsenfeld, G. (2012) Vezf1 protein binding sites genome-wide are associated with pausing of elongating RNA polymerase II. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 2370-2375
14. Gowher, H., Stuhlmann, H., and Felsenfeld, G. (2008) Vezf1 regulates genomic DNA methylation through its effects on expression of DNA methyltransferase Dnmt3b. *Genes & development* **22**, 2075-2084
15. Carmeliet, P. (2000) Mechanisms of angiogenesis and arteriogenesis. *Nature medicine* **6**, 389-395
16. Flamme, I., Frolich, T., and Risau, W. (1997) Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *Journal of cellular physiology* **173**, 206-210
17. Eccles, S. A., Box, C., and Court, W. (2005) Cell migration/invasion assays and their application in cancer drug discovery. *Biotechnology annual review* **11**, 391-421
18. Gasparini, G., Longo, R., Fanelli, M., and Teicher, B. A. (2005) Combination of antiangiogenic therapy with other anticancer therapies: results, challenges, and open questions. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23**, 1295-1311
19. Chung, A. S., and Ferrara, N. (2011) Developmental and pathological angiogenesis. *Annual review of cell and developmental biology* **27**, 563-584
20. Dudley, A. C. (2012) Tumor endothelial cells. *Cold Spring Harbor perspectives in medicine* **2**, a006536

21. Ghirlando, R., Giles, K., Gowher, H., Xiao, T., Xu, Z., Yao, H., and Felsenfeld, G. (2012) Chromatin domains, insulators, and the regulation of gene expression. *Biochimica et biophysica acta* **1819**, 644-651