MODELING AND INHIBITION OF VEGF-C/VEGFR2 ACTIVE SITE

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ABSTRACT

Recent studies have shown that targeting VEGF is a promising anti-cancer treatment since it is known to be responsible for angiogenesis[11]. Blood supply plays a dual role in tumor growth; it supplies oxygen and nutrients, but also carries chemotherapy and other drugs to the tumor[1]. Tumor vasculature is often leaky and has poor blood flow. VEGF antagonism actually improves blood flow and drug access, but probably also improves oxygenation of the tumor[2]. Therefore, inhibiting VEGF binding is likely important in cancer treatment. Studying VEGF inhibition was done mainly with molecular modeling. The active site was characterized with modeling software and VEGF inhibitor targets were designed based on these modeling studies. The designed ligands were optimized using quantum mechanics based computational methods and several homology studies were done to assess the viability of targeting the VEGFR2 protein.

INTRODUCTION

Angiogenesis plays an important role in tumor growth and metastasis[1]. In the early 1970s, Folkman et al.[2] identified a tumor-angiogenesis factor that is mitogenic to capillary endothelial cells in human and animal solid tumors and suggested that blocking this factor might arrest tumors with a tiny diameter (few millimeters). This was later called vascular endothelial growth factor (VEGF).

VEGF is recognized as an essential regulator of normal and abnormal blood vessel growth. It regulates both vascular proliferation and permeability, and it functions as an antiapoptotic factor for newly formed blood vessels. It is expressed in response to hypoxia, oncogenes, or cytokines, and its expression is associated with poor prognosis in several types of cancer.[3]

In mammals, the VEGFs are encoded by a family of genes including VEGF-A, -B, -C, -D, and placental growth factor (PGF).[4] VEGF-A, -B, and PGF are predominantly required for blood vessel formation, while VEGF-C and -D are essential for the formation of lymphatic vessels.[5]

The biological functions of VEGF require binding to receptor tyrosine kinases; VEGFR-1, VEGFR-2, and VEGFR-3.[6] VEGF-A binds to VEGFR-1 and -2, while VEGF-C and -D bind to VEGFR-2 and -3 (Fig 1). [7]

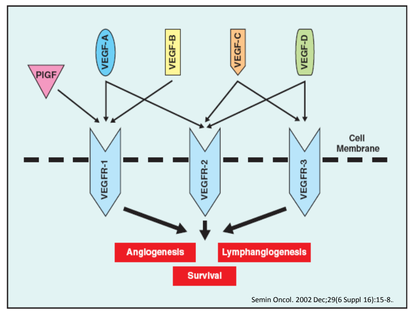

Fig 1. — VEGF Ligand and receptor interactions. PlGF = placental growth factor.

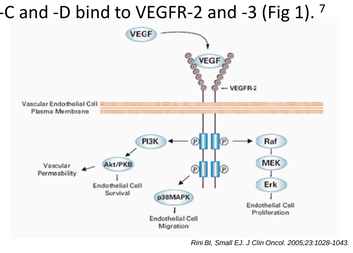

Fig.2: A simplified representation of the downstream cellular effects of the dimerization and activation of the VEGF/VEGFR-2 complex. The ensuing signaling cascade includes the PI3K/Akt pathway, which leads to endothelial cell survival; the p38MAPK pathway, which promotes endothelial cell migration; and the Raf pathway, which induces endothelial cell proliferation. All of these effects promote tumor angiogenesis.

Several signaling pathways are activated by VEGFR2, including the PI 3- kinase/Akt pathway[8] and the classical Ras-dependent signaling cascade impinging on MAP kinases such as ERK1 and ERK2(**Fig 2**). [9,10]

Recent studies have shown that targeting VEGF is a promising anti-cancer treatment since it is known to be responsible for angiogenesis and inhibition of apoptosis.[11,12] Blood supply in tumors has a dual role that includes transporting not only oxygen and nutrients, but also chemotherapy and other drugs. VEGF inhibition has been shown to decrease tumor vasculature permeability in both animal and human tumors, by improving what is normally a leaky tumor vasculature.[13] This has a dual effect of increasing both drug access and oxygen supply to the tumor. Thus, inhibiting VEGF should increases blood flow and improve the clinical outcome of chemotherapy and radiotherapy treatment.[14]

VEGF has also been linked to dormancy induction of tumor cells because it has the ability of signaling inhibition to "damp down" the PI3K-AKT pathway. This should not only potentiate the pro-apoptotic effects of concurrent cytotoxic therapy, but also increase the probability of reverting the disease to dormant status.[15]

METHODOLOGY

Cheminformatics:

1. Spartan ST v2.0.0 , all the designed ligands were geometrically optimized at Hartree-Fock level of theory using 3-21G basic set.
2. Chemoffice2006 (chem3D & chemdraw) to draw chemical structure.
3. Web site: http://zinc.docking.org a free database of commercially-available compounds for virtual screening.

Bioinformatics:

1. Homology studies were done using Blast server provided through NCBI http://blast.ncbi.nlm.nih.gov
2. Prediction of secondary protein structure was done via SOPMA software installed on ExPASy Proteomics tools web site: www.expasy.ch/tools

Active site modeling and ligand fitting:

1. Jmol v12.0.14 a Java based application designed to display various 3D chemical information.
2. Discovery Studio v3.0.0.10321, a software suite of life science molecular design solutions.

Techniques used in Dr Wilkinson research:

1. Anti-phosphotyrosine Western blot.
2. Cell assays (proliferation, cell survival, cell migration) to see how well cells respond to VEGF.
3. To manipulate levels of ECSC "Endothelial Cell-Specific Chemotaxis" protein, they use siRNA (small inhibitory RNA; an oligonucleotide system which will cause degradation of a specific RNA) to reduce its expression or transfection to increase its expression. With cells with altered ECSCR "Endothelial Cell-Specific Chemotaxis Receptor" levels they use their assays to measure sensitivity to VEGF.

RESULTS

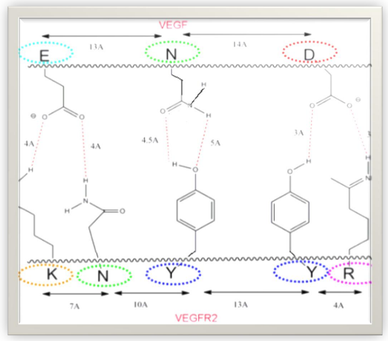

Fig 3: 2-D representation of the most important amino acids involved in VEGF-C/ VEGFR2 interaction with the distances in Å.

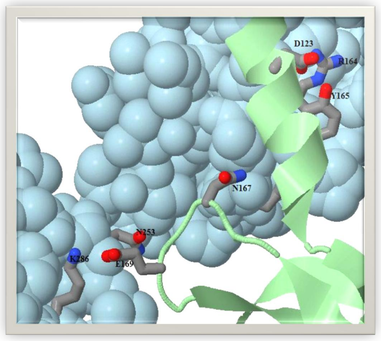

Fig 4: 3-D rendering of the active site of VEGF-C (in blue)/VEGFR2 (in green). Amino acids important to binding are shown.

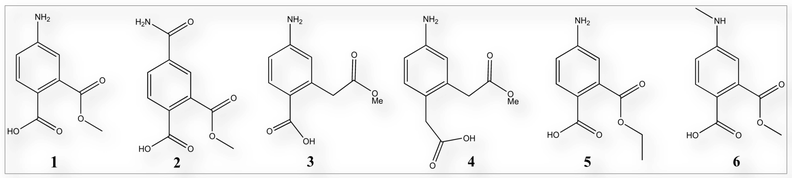

Fig 5: 2-D representation of six designed ligands.

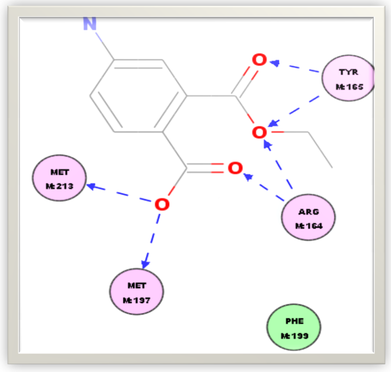

Fig 6: 2-D pharmacophore representation of the active site of VEGFR2.

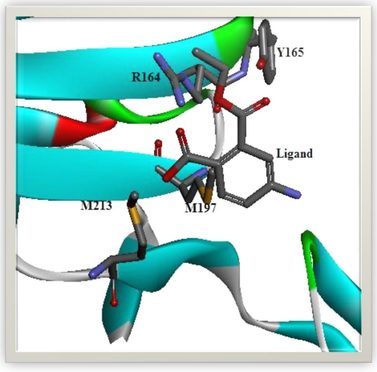

Fig 7: 3-D rendering of ligand 5 in the VEGR2 active site.

Non-human homology study

Protein name	Source	Identities	Homology
vascular endothelial growth factor receptor 2	chicken	97%	98%
kinase insert domain receptor	Taeniopygia guttata	91%	95%
vascular endothelial growth factor receptor 2-like	green anole	74%	85%
similar to VEGFR2	opossum	73%	84%
vascular endothelial growth factor receptor 2-like	Sumatran orangutan	72%	83%
kinase insert domain receptor	chimpanzee	73%	84%
vascular endothelial growth factor receptor 2	pig	73%	84%

Human homology study

Protein name	Source	Identities	Homology
fms-related tyrosine kinase 4	Human	45%	60%
VEGFR3 long form	Human	45%	60%
fms-related tyrosine kinase 1	Human	45%	61%

SOPMA analysis

Secondary structure	Percent
Alpha helix	20.47%
3_{10} helix	0.00%
Pi helix	0.00%
Beta bridge	0.00%
Extended strand	22.55%
Beta turn	4.38%
Bend region	0.00%
Random coil	52.60%
Ambiguous states	0.00%
Other states	0.00%

Active site studies give insight into VEGF-C/VEGFR2 binding interactions. The structure of VEGF-C is an antiparallel homodimer.[15] Two monomers are covalently linked by two disulfide bridges. The links are formed between Cys156 and Cys165.[15] The dimer is then bound between two VEGFR2 receptors. The structure contains two active sites and two hydrophobic sections, with each VEGF-C having one end in the active site and the opposite in a hydrophobic contact. The combination of these interactions should mean tight binding of VEGF-C to VEGFR2.

The active site, shown in Fig. 4, contains both ionic and hydrogen bonding. It is comprised of two main sections that are located close to 27 Å apart. A third interaction, a hydrogen bond, is located approximately half way between the two main areas. A salt bridge in one of the main bonding sites is comprised of Glu169 and Lys286.[15] Hydrogen bonding contacts include Asn167, Tyr194, Asn253, Glu169, Asp123, Arg164, and Tyr165.[15] The effect of two main bonding sites, along with one additional bond, means there is a strong bonding interaction between VEGF-C and VEGFR2.

Homology studies can be used to explore the viability of current and future research on VEGF-C/VEGFR2. Non-human homology studies show that there are several possible animal models to be studied in the future. The high level of homology is promising and this could be a productive way to study possible inhibitor drug targets. Meanwhile, human homology studies suggest that inhibiting VEGF-C binding to VEGFR2 could have adverse side effects. Homologies show that other protein's function could be affected by an inhibitor.

SOPMA analysis shows a perfect match with the X-ray structure where, most of the x-ray structure shows loops, coils, alpha helices, and β-sheets. The active site is located near an alpha helix while there are also significant hydrophobic contacts that are located near β-

FUTURE WORK

Dr. Wilkinson's lab has recently been working on whether a second gene influences how well VEGF can activate its receptor (KDR). His lab measures receptor activation by tyrosine phosphorylation of specific residues, which are needed for downstream signals. In their tests, they found that "their" protein can directly associate with KDR to enhance activation. Their protein, called ECSCR, is controlled via siRNA to reduce or increase its expression. They can then measure sensitivity to VEGF. The Wilkinson group has recently become interested in KDR as being presented on the cell-surface as a complex; which makes drugs targeting the extracellular domain hold promise of altering its sensitivity or signaling output. Future research in anti-VEGF therapies will most likely combine anti-tumor therapy with anti-VEGF treatment at the correct dosage and time.

SUMMARY

The project allowed us to use what we have learned in biochemistry and apply it to a current research project involving an important protein (VEGF). We were able to learn about the metabolic pathways that include VEGF and how it plays a significant role in angiogenesis and inhibiting tumor growth. The most exciting part of this project was to explore the active sites of a protein, and to have a better sense of the nature of the binding. Lastly, work on this project required us to learn some very useful software and techniques that can be applied in a wide array of fields.

REFERENCES

1. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol. 2002 Dec;29(6 Suppl 16):15-8.
2. Folkman J, Merler E, Abernathy C, et al. Isolation of a tumor factor responsible for angiogenesis. J Exp Med. 1971;133:275-288.
3. Shweiki D, Itin A, Soffer D, et al. Vascular endothelial cell growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 1992;359:843-845.
4. Tammela T, Enholm B, Alitalo K, et al. The biology of vascular endothelial growth factors. Cardiovasc Res. 2005;65:550-563.
5. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. Physiol Rev. 2002;82:673-700.
6. Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. The role of VEGF receptors in angiogenesis; complex partnerships. Cell Mol Life Sci. 2006;63:601-615.
7. Errico M, Riccioni T, Iyer S, et al. Identification of placental growth factor determinants for binding and activation of Flt-1 receptor. J Biol Chem. 2004;279: 43929-43939. Epub 2004 Jul 21.
8. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem. 1999;274:16349-16354.
9. Meadows KN, Bryant P, Pumiglia K. Related Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J Biol Chem. 2001;276:49289-49298. Epub 2001 Oct 26.
10. Guo DQ, Wu LW, Dunbar JD, et al. Tumor necrosis factor employs a protein-tyrosine phosphatase to inhibit activation of KDR and vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem. 2000;275:11216-11221.
11. Argraves, W. S., & Drake, C. J.. Genes critical to vasculogenesis as defined by systematic analysis of vascular defects in knockout mice. The Anatomical Record. Part A, Disc. Mol. cell biol. 2005;286(2), 875–884.
12. Jain, R. K., et al.. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat. Clin.Pract oncol. 2006;3(1), 24–40.
13. Lev, D. C., et al.. Exposure of melanoma cells to dacarbazine results in enhanced tumor growth and metastasis in vivo. J Clin Oncol. 2004;22(11), 2092–2100.
14. Ravi, D., et al.. Apoptosis, angiogenesis and proliferation: Trifunctional measure of tumor response to radiotherapy for oral cancer. Oral oncol. 2001;37(2), 164–171.
15. Townson, J. L., & Chambers, A. F. Dormancy of solitary metastatic cells. Cell Cycle. 2006; 5(16), 1744–1750.
16. Leppanen V, Prota A, Jeltsch M, et al. Structural determinants of growth factor binding and specificity by VEGF receptor 2. Proc. Natl. Acad. Sci. USA. 2010; 107: 2425-2430.

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