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Identification of novel angiogenesis inhibitors

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Abstract—Vascular endothelial growth factor (VEGF) is a key stimulant of angiogenesis, which is the process of generating new capillary blood vessels. Inhibition of the vascular endothelial growth factor receptor (VEGFR) kinase is known to result in blockage of angiogenesis. A pharmacophore was developed based on the binding of ATP to the hinge region of the kinase domain of VEGFR and a database search of 18,000 compounds was conducted. Selected hits were assessed for their ability to limit the induction of web-like network of capillary tubes by the human umbilical vascular endothelial cells. Two compounds (**1** and **4**) showed good inhibitory ability to prevent sprouting and closed polygon formation of the tubular networks, promising them to be lead compounds. Compound **4** showed 60% inhibition at 0.05 μM.

Angiogenesis, the process by which new blood capillaries are formed from pre-existing blood vessels, occurs during development and tissue regeneration, wound healing, chronic inflammatory conditions, and in diabetic retinopathy.[1] Tumor angiogenesis is a critical process required by most solid tumors to support their localized growth and metastatic dissemination.[2,3] Vascular endothelial growth factor (VEGF) is one of the most important factors involved in promoting tumor angiogenesis and is secreted by almost all solid tumors and tumor-associated stroma in response to hypoxia.[4,5] Binding of VEGF to its receptors triggers kinase activation through tyrosine phosphorylation and begins the signaling cascade that initiates angiogenesis. VEGF appears to play a multitude of indispensable roles, including increase in vascular permeability, which in turn may facilitate tumor dissemination via circulation;[6,7] inhibition of endothelial cell apoptosis by inducing expression of the survival gene BCL-2[8] promote tumor growth and also lead to resistance and further to cytotoxic chemotherapy. Potential therapeutic approaches to inhibit angiogenesis include neutralizing antibodies against VEGF, soluble receptors, ribozymes directed against VEGF receptor, and VEGFR tyrosine kinase inhibitors that target the intracellular signal transduction. A variety of anti-angiogenesis therapies directed against the VEGFR kinase have been a promising and well-validated therapeutic approach under active evaluation of their safety and efficacy in multiple clinical trials.[9,10] Most of the small molecule inhibitors of VEGFR kinase are ATP competitive, by binding to the ATP-pocket of the kinase domain. Among the first generation, VEGFR kinase inhibitors were the indolinones, SU5416, and SU6668, which disappointed in clinical development due to their adverse reactions in clinical trials.[11] The promising effects on the growth of colon tumors with PTK787[12,13] further encourage the development of second generation VEGFR kinase inhibitors. New generations of anti-VEGFR compounds based on a variety of chemical scaffolds are now emerging, as exemplified by AAL993,[14] CEP-7055,[15] and CP-547632.[16]

Intrigued by the importance of VEGFR in solid tumors, we embarked on the search for new lead compounds that could act as potential inhibitors of angiogenesis. On the basis of the known VEGFR kinase inhibitors, a series of basic chemical scaffolds were built and a

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similarity search of our in-house database was carried out. The compounds that emerged from the search were analyzed for their angiogenesis inhibiting activity using an in vitro angiogenesis assay. Two of the compounds (**1** and **4**) with 1,3-dioxo-2,3-dihydro-1*H*-isoindol-amide scaffold showed excellent angiogenesis inhibitory activity at 10 μM and the compound **4** showed good inhibition at 0.05 μM concentration.

The available crystal structure of the apo VEGFR-2/KDR has several missing residues including the connecting loops.[17] Hence, the homology model was generated using the program Modeller-6v2.[18] The input alignment for the Modeler was obtained with ClustalW,[19] based on the sequence of the human VEGFR-2/KDR kinase domain (PDB code = 1VR2).[17] The homology model was built using the tyrosine kinase domain of fibroblast growth factor receptor 1 in complex with SU4984 (PDB code = 1AGW) as a template structure.[20] The missing loops were built using the 'loop model' building option in the Modeller-6v2.[21] The model was refined further by energy minimization routine of DISCOVER module of INSIGHT II Accelrys Inc., San Diego, CA, USA. The quality of the refined model was checked with PROCHECK.[22] The VEGFR-2 cavity residues were identified based on the available crystal structure-binding model for staurosporine and SU4984,[20] known ATP-competitive inhibitors. Unity[23] module of Sybyl 6.9 was used for the pharmacophore-based similarity searches. To define the pharmacophore, the acceptor (A) and donor (D) sites were defined with distance constraints, and it is measured from the backbone residues Cys919 and Glu917 of the VEGFR-2 protein kinase (Fig. 1). By using receptor site module, exclusion spheres were defined up to 5 Å region from the pharmacophore site to get appropriately docked structures. A database search based on the above pharmacophore generated a set of 769 hits. Further, the UNITY 2 D similarity search was carried out using core structures depicted by some of the VEGFR inhibitors such as SU5416 and CEP-7055. The 34 hits (ligands) obtained thus were docked to VEGFR kinase domain.

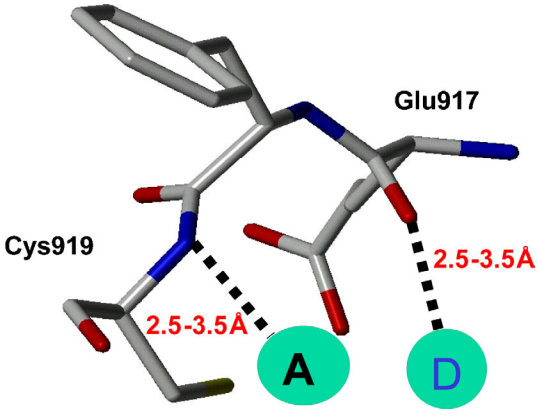

Figure 1. Backbone residues defined as pharmacophores, depicting the two residues (Glu917 and Cys919) with which the ligands interact by making hydrogen bonds. A is the acceptor atom from ligand and D is the donor. The hydrogen bond distances are shown by dashed lines and the distance ranges utilized for database search are also shown.

Initial geometric optimizations of 34 ligands were carried out using the standard MMFF94 force field, with a 0.001 kcal/mol energy gradient convergence criterion and a distance-dependent dielectric constant employing Gasteiger charges. Additional geometric optimizations were performed using the semi-empirical method molecular orbital package (MOPAC).[24] FlexX program was used to dock 34 structures, which are identified from similarity search. All the predicted binding models were energy minimized using the DISCOVER module of Insight II.

Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Co. (East Rutherford, NJ, USA) and maintained in endothelial growth medium (EGM) supplemented with 2% FBS, 0.1% EGF, 0.1% hydrocortisone, 0.1% GA-1000, and, 0.4% BBE. Morphogenesis assay on Matrigel was performed according to the manufacturer's instructions (Chemicon International). The ECMatrix™ kit consists of laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, and nidogen. It also contains various growth factors (TGF-β, FGF) and proteolytic enzymes (plasminogen, tPA, and MMPs) that are normally produced in EHS tumors. The incubation condition was optimized for maximal tube-formation as follows: 50 μl of EC Matrix™ was suitably diluted in the ratio 9:1 with 10× diluent buffer and used for coating the 96-well plate. The coated plates were incubated at 37 °C for 1 h to allow the Matrix solution to solidify. In the meantime, the HUVEC that were cultured for 24 h in EGM with 2% FBS was trypsinised and re-suspended in the growth medium for cell counting. After 1 h pre-incubation of the plate with Matrix solution, the HUVEC were plated at 10^4 cells/well in the absence or in the presence of different VEGFR inhibitors (1 and 10 μM). After 8 h of incubation at 37 °C, the three-dimensional organization (cellular network structures) was examined under an inverted photomicroscope. Each treatment was performed in triplicates.

Protein kinases are highly conserved at the ATP-binding site, which consists of a hydrogen-bonding region called the hinge region. Inhibitors often achieve hydrogen bonding to the hinge region by means of a bi-dentate donor–acceptor system present in their structure.[1] To identify the small molecular inhibitors for VEGFR activity, in our computational methodology, we initially used pharmacophore search based on donor–acceptor interactions with the backbone residues Cys919 N–H and Glu917 C=O of VEGFR kinase (Fig. 1). Using this simple pharmacophore model, we searched a database that contains 18,000 structures using the Unity search. At the end, a total of 769 compounds were identified from the database that met the pharmacophore requirements as specified in Figure 1. Accordingly, it is anticipated that compounds satisfying those pharmacophoric points will have a high probability of being biologically active, and the identified 769 compounds are structurally diverse. Screening 769 compounds using the in vitro angiogenesis assay would be quite time-consuming; our goal was to identify good inhibitory compound from our dataset of 769 structures, and

similarity search was carried out using the isoindolinone moiety. A total of 34 compounds were identified from the similarity search, which were docked onto the ATP-binding site of VEGFR-2 kinase homology model built in our laboratory (Fig. 2). Two sets of compounds **1**–**3** and **4**–**6** representing (1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl)-amide and (1,3-dioxo-2,3-dihydro-1*H*-isoindol-5-yl)-amide (Fig. 3; referred to in this paper as pthalimide) were taken up on the basis of the binding features and tested for angiogenesis activity. The initial hit list consisted of another series of compounds that had a substituted 5,6-diamino-2-mercapto-3*H*-pyrimidin-4-one core. Due to the novelty of the core and their viability for multiple hydrogen bonds with the protein, two compounds **7** and **8** (Fig. 3) were chosen with a representative substitution pattern.

The efficiency of these compounds in inhibiting the VEGFR kinase was measured by their ability to stabilize or slow down the progression of angiogenesis. There are many assays in existence that are known to be representative of in vivo angiogenesis.[25] A study of formation of the three-dimensional web-like structures of interconnected cells (cords) by the endothelial cells is one of the popular and widely used qualitative assays.[26] The formation of the cellular networks progresses in a stepwise manner with the initial migration and alignment of cells, development of capillary tube like structures, sprouting

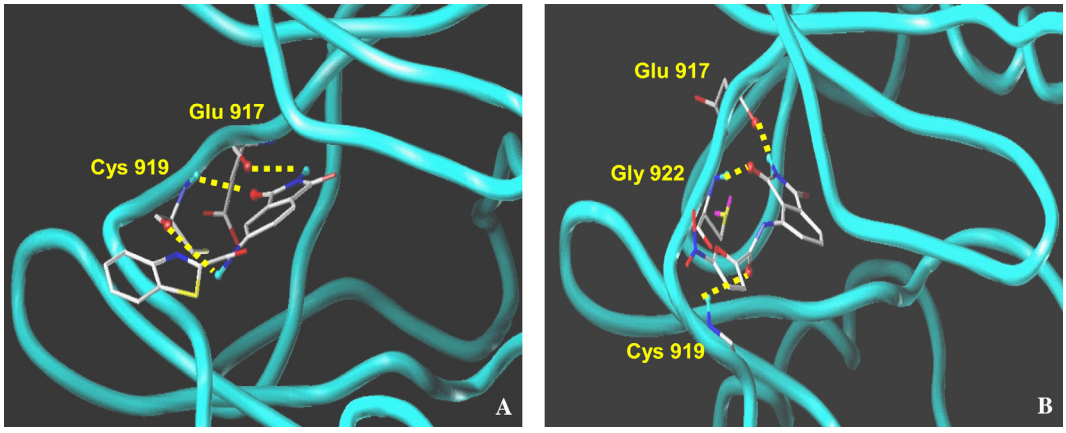

Figure 2. Binding mode of compound **1** (A) and compound **4** (B) with VEGFR kinase domain. The hydrogen-bonding interactions are represented as yellow dotted lines; the ligand and interacting residues are shown as ball and stick models.

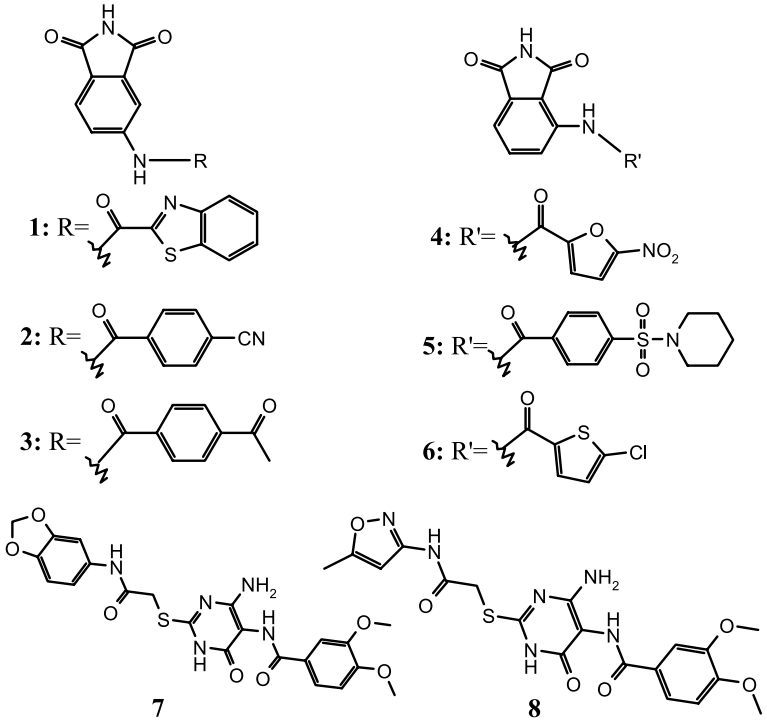

Figure 3. Structures of compounds **1**–**8**.

of new branches, and finally formation of cellular networks. The compounds **1**–**8** were subjected to the above assay to understand their ability to inhibit the activity of endothelial cells. The results are depicted in Figure 4 and Table 1. As can be seen, compounds **1** and **4** inhibit progression of angiogenesis to a major extent at 10 μM concentration. The cells do not migrate and align, and they do not sprout branches for network formation. Compounds **2**, **3**, **5**, and **6** also show inhibition of angiogenesis, but to a lesser extent. Compound **7** showed lesser inhibition of angiogenetic process, perhaps, showed only the final stage of the process. Compound **8** has negligible inhibitory effect. The effectiveness of angiogenesis inhibition shown by compounds **1** and **4** present these as the most promising lead compounds. They make the required two hydrogen bonds with the hinge region residues Glu917 and Cys919 of VEGFR kinase. The dose–response curve for the compound **4** is shown in Figure 5. This compound has 60% inhibition at 0.05 μM concentration. Compound **1** makes additional hydrogen bond with Cys919 by the side chain amide –NH and the terminal amino group (Fig. 2A). One additional hydrogen bond is formed between the side chain amide carbonyl of compound **4** and Gly922 of the protein (Fig. 2B). These additional interactions could be the contributing factors for the better inhibition of angiogenesis.

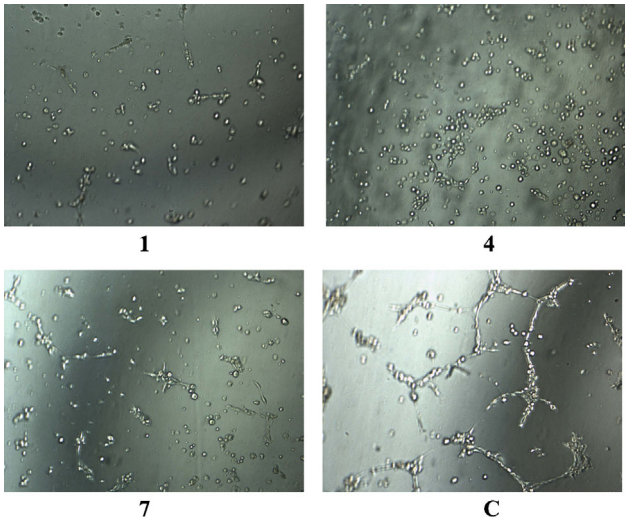

Figure 4. In vitro angiogenesis assay of compounds **1**, **4**, and **7**. The control is depicted as **C**.

Table 1. Scoring of the angiogenesis assay illustrated in Figure 4

Compound	Concentration 10 μm
1	++++
2	+++
3	++
4	+++++
5	+++
6	+++
7	++
8	+

The score is based on the extent of cellular networks: (+++++) = individual cells, well separated, (++++) = cells begin to migrate and align, (+++) = cells lineup but do not sprout, (++) = visible sprouting, and (+) = closed polygons begin to form.

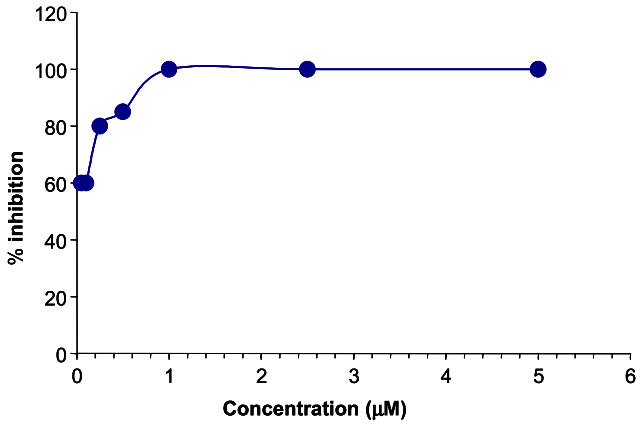

Figure 5. Dose–response curve for the compound **4**.

Novel cores have been identified containing pthalimide and 5,6-diamino-2-mercapto-3*H*-pyrimidin-4-one. Further modifications of these cores will be carried out for obtaining more potent angiogenesis inhibitors. Different substitutions and heterocyclic ring extensions for the side chain will be studied to improve the potency of these lead compounds. Furthermore, it is necessary to verify that these compounds inhibit directly the function of VEGFR kinase in order to establish a connection between VEGFR kinase targets and angiogenesis.

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