Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4551–4556
Bioorganic & Medicinal Chemistry Letters

In-silico fragment-based identification of novel angiogenesis inhibitors

Sivanesan Dakshanamurthy,[a,b,*] Min Kim,[a] Milton L. Brown[a,b] and Stephen W. Byers[a]

[a]*Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, USA*
[b]*Drug Discovery Program, Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, USA*

Received 10 April 2007; revised 27 May 2007; accepted 30 May 2007
Available online 15 June 2007

Abstract—Inhibition of vascular endothelial growth factor receptor-2 (VEGFR2) kinase blocks angiogenesis, the process of generating new capillary blood vessels that are important in tumor growth. To identify small molecule inhibitors of the VEGFR2 kinase, we undertook a computer assisted fragment-based screening that used 3-D structural models of the VEGFR2 kinase, and hinge region as a fragment anchor point. Seven novel non-cytotoxic compounds were identified which limited the induction of capillary networks by human umbilical vein endothelial cells in the low micromolar range.

Angiogenesis, the process of new blood vessel formation by capillary sprouting from pre-existing vasculature, is important in many diseases and is required for tumor growth and metastasis.[1,2] Anti-angiogenesis therapy is an important cancer treatment option as it is less likely to result in resistance than therapies that affect the growing tumor directly.[1] The vascular endothelial growth factor receptor (VEGFR) pathway has become a major focus of drug development in the field of oncology. There are several members of the VEGF family (i.e., A, B, C, and D) and several VEGF receptors (VEGF receptor-1 or Flt-1, VEGF receptor-2, VEGF receptor-3 or Flt-4).[3] Importantly, activation of VEGFR2 alone is essential for new vessel initiation in the early stages of angiogenesis through induction of proliferation, migration, and survival of endothelial cells.[4] Consequently, inhibition of VEGFR2 signal-transduction may provide a means of stabilizing or slowing down the progression of solid tumors. Several approaches have been used to target the VEGF/VEGFR2 pathway. The most common of these is the use of ATP analogs that are selective for the inhibition of the tyrosine kinase activity of VEGFR2. The ATP binding site of VEGFR2 can accommodate a number of chemically and structurally diverse molecular scaffolds.[5] In this study, we applied computer-based fragment screening to identify diverse and new scaffolds of VEGFR2 inhibitors. Such fragment screening strategies are of increasing importance in drug discovery.[6] A series of basic chemical fragments were screened against the VEGFR2 kinase domain and a 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. Seven of the compounds showed significant angiogenesis inhibitory activity at 10 μM.

As the available crystal structure of the VEGFR2 (PDB: 1YWN) has several missing residues including F1047 (see below), we constructed a homology model using the program Modeller.[7] The input alignment for the Modeller was obtained with ClustalW[8] based on the sequence of the human VEGFR2 kinase domain (PDB: 1VR2). We then redocked the known ligand (LIF, pyrimidin-5-yl]phenyl-*N*′-[2-fluoro-5-(trifluoromethyl)phenyl]urea) (LIF VEGFR2 structure PDB:1YWN) into the predicted VEGFR2 structure. Because of unfavorable steric interactions with F1047 and the ligand, we remodeled the predicted structure based on the EGFR crystal structure (PDB: 1M17). The structure was energy minimized for 10,000 cycles with a consistent valence force field (cff91) using the DISCOVER module of INSIGHT II (Accelrys Inc., San Diego, CA, USA). We again compared this new model with the LIF.VEGFR2 structure to ensure that ATP binding site residue side chains are in the proper orientations

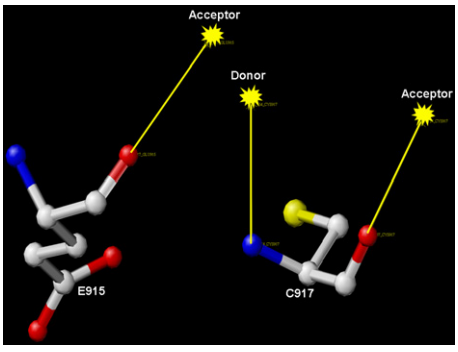
Keywords: Fragment-screening; Docking; Molecular dynamics; Angiogenesis inhibitors; VEGFR2.
* Corresponding author. E-mail addresses: sd233@georgetown.edu; sivanesan_dk@yahoo.com

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.05.104

after remodeling. This final predicted model was used for docking simulations. The best complexes were energy minimized followed by brief molecular dynamic (MD) simulations to relax the structure.[9] The two water molecules within the VEGFR2 ATP active site were included in the simulations. The UNITY module of Sybyl 7.2 (Tripos, Inc., St. Louis) was used for the chemical database searches and the FlexX program (Tripos, Inc.) was used for docking simulations.

Kinase inhibitors usually interact with the hinge region using a bi-dentate donor–acceptor system present in their structure.[10] We reasoned that compounds containing fragments forming H-bonds at the hinge region are like to be biologically active. We used fragment-based screening in an attempt to identify molecular anchors at the hinge region. We focused our fragment screening on the hinge region by providing pharmacophore constraints (Scheme 1) on the hinge-region residues E915 and C917 of the reported VEGFR2 structure as described below: to define the pharmacophore, the acceptor and donor sites were located at the backbone atoms of C919 and E917 (Scheme 1). We applied the following constraints: (1) H-bond acceptor is essential; (2) H-bond donor is essential; (3) H-bond acceptor and donor are essential. These pharmacophore constraints were assigned prior to the docking simulations for fragment screening using FlexX-Pharm, Sybl7.2. We used our in-house fragment database that contains ~19,112 unique lead-like organic fragments collected from the literature, Web sources, and commercial chemical companies. As most ATP kinase inhibitors possess a monocyclic or heterocyclic planar ring, we restricted our search to fragments that contained five or six member mono-cyclic or hetero cyclic rings and that make at least one H-bond with the hinge region. For a successful placement, a ligand needed to bind to at least one of the three selected pharmacophore points (Scheme 1).

Our fragment screening yielded several new scaffolds (highlighted in red in Table 1) that potentially bind to the hinge region. We next initiated a search for compounds containing these 'fragment hits' through our database that comprises ~82 million organic molecules. We have several physico-chemical filtering schemes such as Lipinski rule of five, chemical stability, reactive groups, synthetic feasibility, polar surface area, rotatable bonds, etc. After applying this in-house physico-chemical filtering schemes we obtained 10,083 hits. Then, we initiated docking simulations to screen these compounds against VEGFR2. Self-docking of LIF ligand gave a score (FlexX energy) of −29.5 kcal/mol. Thus, an arbitrary cutoff score was chosen as −20 kcal/mol, to take into account limitations that vary from the rigid representation of the receptor and inaccuracies in the scoring function. We selected 613 compounds (from a total of 43,114) that scored better than 20 kcal/mol and made at least one hydrogen bond with the hinge-region backbone. These were clustered according to chemical similarity and visual inspection, resulting in a final list of 412 compounds. Screening such large dataset using the in-vitro angiogenesis assay would be quite time consuming. As our goal was to identify new and diverse fragment cores, we selected the 81 best compounds that have unique structural cores when compared to known VEGFR2 inhibitors. We obtained some of these compounds from the National Cancer Institute and from commercial vendors.

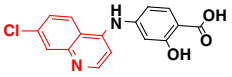
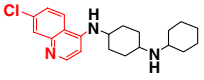
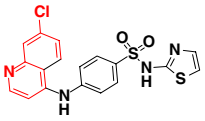
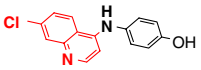
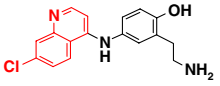
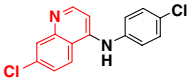
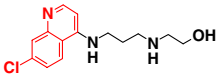
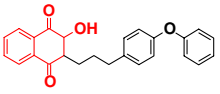
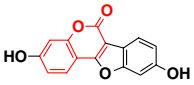
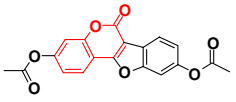
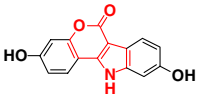
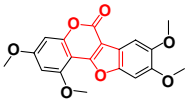
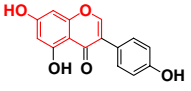
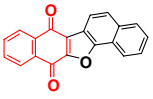


Scheme 1. Hinge-region backbone atoms of E915 and C917 considered to define constrained pharmacophore points with which ligand interacts by making H-bonds. For successful placement a ligand needs to bind to at least one of the three pharmacophore points assigned using FlexX-Pharm. Yellow stars indicate pharmacophore H-bond constraints used during docking.

The efficiency of these compounds in inhibiting the VEGFR2 kinase was measured by their ability to stabilize or slow down the progression of angiogenesis. Compounds **1**–**17** were tested for their ability to block angiogenesis progression in the in-vitro angiogenesis assay using HUVEC. In this assay, 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 of new branches, and finally formation of cellular networks.[11] The HUVEC assay[12] results are displayed in Figure 1 and Table 1. Compounds **1**–**4**, **6**, **11**, and **14** markedly inhibit network formation at 10 μM. The cells do not migrate and align, and they do not sprout branches or form networks.

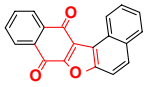
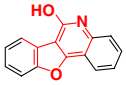
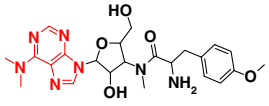
Compounds **8**, **9**, **10**, **12**, **15**, and **16** also show inhibition of in-vitro angiogenesis and compounds **5**, **7**, and **13** only inhibited the later stages of network formation. Compound **17** was ineffective. The cytotoxicity IC_{50}[13] values of VEGFR2 inhibitors in HUVEC are listed in Table 1, Figures 2 and 3. Among the effective inhibitors tested, **1**, **3**, **4**, **6**, **8**, **13**, and **14**, showed low cytotoxicity (>50 μM) to HUVEC. This means that these compounds are not toxic to HUVEC within the tested range. These VEGFR2 inhibitors completely inhibited HUVEC network formation at concentrations 10× less than those that inhibited HUVEC proliferation or viability by 50%. These results suggest that anti-angiogenesis effects are not from non-specific cytotoxic effect but from specific VEGFR2 receptor inhibition. Although these experiments point to a specific effect of the inhibitors on angiogenesis itself (without effects on proliferation or apoptosis) several reports indicate that induction of apoptosis in HUVEC is also a significant mechanism of anti-angiogenesis.[14] Consequently, even though the VEGFR2 inhibitors used in the present

Table 1. Cytotoxicity,[13] scoring of angiogenesis assay[11,12] results, and the structures of investigated compounds

Compound	Cytotoxicity IC_{50} (μM)	Scoring of angiogenesis assay at 10 μM	Structure
1	90	+++++	
2	13	++++	
3	74	++++	
4	82	++++	
5	ND	++	
6	88	++++	
7	ND	++	
8	75	+++	
9	ND	+++	
10	ND	+++	
11	ND	++++	
12	ND	+++	
13	88	++	
14	63	++++	

(*continued on next page*)

Table 1 (*continued*)

Compound	Cytotoxicity IC_{50} (μM)	Scoring of angiogenesis assay at 10 μM	Structure
15	ND	+++	
16	ND	+++	
17	100	+	

A non-standardized scoring system was used for comparing anti-angiogenic effect. The values were assigned based on the extent of inhibition on network formation: (1) individual cells well separated (score: +++++), (2) cells begin to migrate and align themselves (score: ++++), (3) cells begin to line but no sprouting (score: +++), (4) visible sprouting (score: ++). ND, not determined.

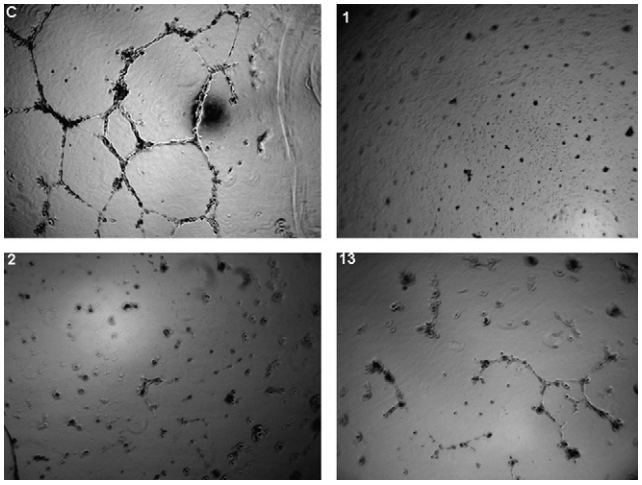


Figure 1. In-vitro angiogenesis assay results of compounds **1**, **2**, and **13**, where control denoted as 'C'.

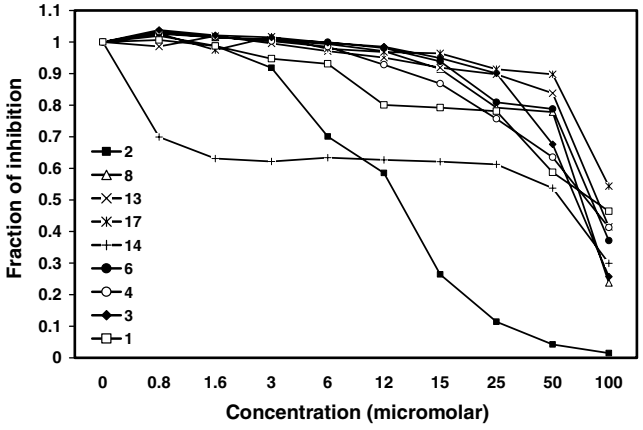


Figure 2. CytotoxicityIC_{50}[13] values for compounds **1**–**4**, **6**, **8**, **13**, **14**, and **17**.

study did not show significant cytotoxicity, inducing apoptosis specifically in endothelial cells undergoing angiogenesis could actually constitute a viable therapeutic approach.

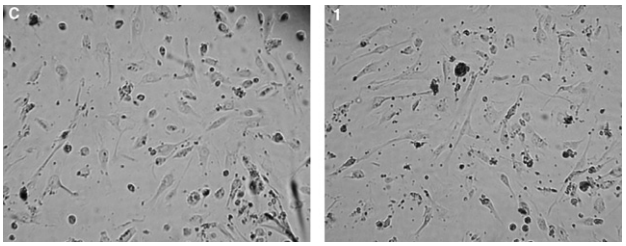


Figure 3. In-vitro cytotoxicity cell viability assay results for compound **1** and control denoted as 'C'.

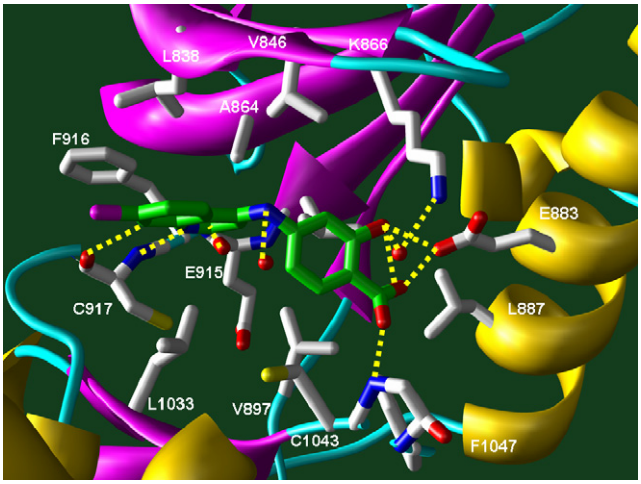


Figure 4. Predicted binding mode of compound **1** identified in our fragment-based screening.

The cytotoxicity of compound **1** is 90 μM (Table 1), that is, cell growth inhibition not seen up to 90 μM (Fig. 3). Figure 4 depicts the proposed binding mode for compound **1** after MD simulations. This compound makes the required H-bonds with the hinge-region residues; one N···N–H H-bond with C917 and two C–H···O H-bonds with E915 and C917. Conformational rearrangement of K836, E883, the two water molecules, and the compound **1** led to a H-bond between the hydroxyl and –COOH groups of compound **1** and K836, E883, F1047 in addition to an intra-molecular

H-bond between these acidic groups. Two water mediated H-bonds have been observed one with the imino group and other with COOH-group of compound **1**. The hydrophobic moiety was surrounded by bulky groups such as L838, K866, V846, A864, L887, V897 F916, and L1033. These additional interactions could be contributing factors for improved angiogenesis inhibition. This molecular model will be useful for further lead optimization.

In summary, in this study we applied computer-based fragment screening with pharmacophore constraints followed by docking simulations to identify novel non-cytotoxic angiogenesis inhibitors effective in the low micromolar range. Different substitutions and heterocyclic ring extensions for the side chains may improve the potency of these fragment derived lead compounds. To confirm direct inhibition of the function of VEGFR2 kinase, in-vitro studies are in progress using specific phospho-VEGFR2 antibody. Finally this computer-based fragment-screening approach may provide advancement to the drug discovery.

Acknowledgments

We gratefully acknowledge the National Cancer Institute (NCI) for providing some of the compounds investigated in this study. S.D. acknowledges Advanced Biomedical Computing Center of the National Cancer Institute, Frederick, USA, for allocation of computing time and staff support.

References and notes

1. (a) Arap, W.; Pasqualini, R.; Ruoslahti, E. *Curr. Opin. Oncol.* **1998**, *10*, 560; (b) Battegaye, J. *J. Mol. Med.* **1995**, *73*, 333.
2. (a) Folkman, J. *N. Engl. J. Med* **1971**, *285*, 1182; (b) Miller, K.; Sledge, G. W. *Breast Cancer Management Application of Clinical and Translational Evidence to Patient Care*, 2nd ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2003, 287.
3. (a) Leung, D. W.; Cachianes, G.; Kuang, W. J.; Goeddel, D. V.; Ferrara, N. *Science* **1989**, *246*, 1306; (b) Toi, M.; Hoshina, S.; Takayanagi, T.; Tominaga, T. *Jpn. J. Cancer Res.* **1994**, *85*, 1045; (c) Mattern, J.; Stammler, G.; Koomagi, R.; Wallwiener, D.; Kaufmann, M. *Anticancer Res.* **1997**, *17*, 621; (d) Ferrara, N.; Winer, J.; Burton, T.; Rowland, A.; Siegel, M. *J. Clin. Invest.* **1993**, *91*, 160; (e) Kondo, S.; Asano, M.; Suzuki, H. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 1234; (f) Asano, M.; Yukita, A.; Matsumoto, T.; Kondo, S.; Suzuki, H. *Cancer Res.* **1995**, *55*, 5296; (g) Warren, R. S.; Yuan, H.; Matli, M. R.; Gillett, N. A.; Ferrara, N. *J. Clin. Invest.* **1995**, *95*, 1789; (h) Jones, A.; Harris, A. L. *Cancer J. Sci. Am.* **1998**, *4*, 209.
4. (a) Karkkainen, M. J.; Petrova, T. V. *Oncogene* **2000**, *19*, 5598; (b) Ortega, N.; Jonca, F.; Vincent, S.; Favard, C.; Ruchoux, M. M. *Am. J. Pathol.* **1997**, *151*, 1215; (c) Ogawa, S.; Oku, A.; Sawano, A.; Yamaguchi, S.; Yazaki, Y. *J. Biol. Chem.* **1998**, *273*, 31273; (d) Meyer, M.; Clauss, M.; Lepple-Wienhues, A.; Waltenberger, J.; Augustin, H. G. *EMBO J.* **1999**, *18*, 363; (e) Zhang, W.; Ran, S.; Sambade, M.; Huang, X.; Thorpe, P. E. *Angiogenesis* **2002**, *5*, 35; (f) Takahashi, Y.; Kitadai, Y.; Bucana, C. D.; Cleary, K. R.; Ellis, L. M. *Cancer Res.* **1995**, *55*, 3964.
5. (a) Miao, R. Q.; Agata, J.; Chao, L.; Chao, J. *Blood* **2002**, *100*, 3245; (b) Laird, A. D.; Vajkoczy, P.; Shawver, L. K.; Thurnher, A.; Liang, C. *Cancer Res.* **2000**, *60*, 4152; (c) Wedge, S. R.; Ogilvie, D. J.; Dukes, M.; Kendrew, J.; Curwen, J. O. *Cancer Res.* **2000**, *60*, 970; (d) Morin, M. J. *Oncogene* **2000**, *19*, 6574.
6. (a) Rees, D. C.; Congreve, M.; Murray, C. W.; Carr, R. *Nat. Rev. Drug Discov.* **2004**, *3*, 660; (b) Fattori, D. *Drug Discov. Today* **2004**, *9*, 229; (c) Verdonk, M. L.; Hartshorn, M. J. *Curr. Opin. Drug Discov. Dev.* **2004**, *7*, 404; (d) Rummey, C.; Nordhoff, S.; Thiemann, M.; Metz, G. *Biol. Org. Med. Chem. Lett.* **2006**, *16*, 1405; (e) Oblak, M.; Golic, S.; Kotnik, M.; Jerala, R.; Filipič, M.; Šolmajer, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5207.
7. Sali, A.; Blundell, T. L. *J. Mol. Biol.* **1993**, *234*, 779.
8. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673.
9. Minimization was carried out using DISCOVER, Insight II (Accelrys, Inc. San Diego) with a cff91 force field, cutoff for non-bonded interaction energies set to ∞ and dielectric constant set at 4. All atoms within 7.0 Å of the inhibitor were allowed to relax during the minimization. Brief MD simulations were also performed in the NVE ensemble consisted of an initial equilibration of 25 ps and followed by a production run of 100 ps dynamics at 300 K. The final complex structure at the end of the MD simulations was subjected to minimization. A distance-dependent dielectric constant and non-bonded distance cutoff of 12 Å were used. MD simulations were performed using the AMBER8.0 package with the General Amber Force Field (GAFF) and RESP charge models.
10. Folkman, J. *Nat. Med.* **1995**, *1*, 27.
11. (a) Montesano, R.; Orci, L.; Vassalli, P. *J. Cell Biol.* **1983**, *97*, 1648; (b) Vailhe, B.; Vittet, D.; Feige, J.-J. *Lab. Invest.* **2001**, *81*, 439.
12. Chemicals were freshly dissolved in 50 μl of DMSO and diluted with endothelial growth medium (EGM) to final concentration of 1 mM. The highest concentration of DMSO is 0.1%.
    Cell culture: 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. Little more description may be needed.
    Angiogenesis assay: morphogenesis assay on Matrigel was performed according to the manufacturer's instructions (Chemicon International). The ECMatrixTM kit consists of laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, and nidogen. It also contains various growth factors (TGF-b, 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 TM 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 1hr to allow the Matrix solution to solidify. In the meantime, the HUVEC that were cultured for 24 h in EGM with 2% FBS were trypsinized 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 5×10^3 cells/well in the absence or in the presence of different drug (1–100 μM). After 18 h of incubation at 37 °C, the three-dimensional organization (cellular network structures)

was examined under an inverted photomicroscope. Each treatment was performed in triplicate.

13. Active growth inhibition effect of compounds on HUVEC: 5000 cells/well were seeded in 96-well culture plate to ensure the cells are in exponential growth at the time of VEGFR2 inhibitor treatment. A wide range of dose titration (0, 1.6, 3.1, 6.2, 12.5, 25, 50, and 100 μM) of VEGFR2 inhibitors was applied in the study and the cells were incubated with or without inhibitors for 24 h. MTT assay and Cell Titer-Glo assay were used to evaluate the effects of inhibitors on the cellular growth of HUVEC.
Cell viability assay: cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Five thousand cells were seeded into a well of 100 μl EGM complete media for 24 h at 37 °C, 5% CO_2. The cells were then treated with either DMSO or drugs for 24 h. Cell confluence was about 70% at beginning of drug treatment. Viability of the cells was tested by MTT assay according to manufacturer's instructions. Ten microliters of stock solution of MTT (5 mg/ml) was added to each well with 90 μl of EGM and incubated for 3 h at 37 °C. The incubation medium was removed and the formazan crystals were dissolved in 100 μl of solution of DMSO. MTT reduction was quantified by measuring the light absorbance with a microplate counter (VICTOR2—Wallac) at 570 nM. Each treatment was performed in triplicate. Viability of the cells was also tested by Cell Titer-Glo assay (Promega Catalog No. G7571) according to the instructions provided by the manufacturer.
14. (a) Xiao, D.; Singh, S. V. *Cancer Res.* **2007**, *67*, 2239; (b) Lin, H. P.; Kulp, S. K.; Tseng, P. H.; Yang, Y. T.; Yang, C. C.; Chen, C. S.; Chen, C. S. *Mol. Cancer Ther.* **2004**, *3*, 1671.