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DEVELOPMENT OF A HIGH THROUGHPUT ASSAY FOR VEGFR2 INHIBITION

A Major Qualifying Project Report

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ABSTRACT

Over-expression of Vascular Endothelial Growth Factor-A (VEGF-A) can lead to a variety of human diseases, including different forms of cancer, psoriasis, and arthritis. The goal of this project was to develop a cell culture-based assay using human umbilical vein endothelial cells (HUVECs) to identify potential inhibitors of the VEGF-Receptor-2 (VEGFR2) and potential blockers of angiogenesis. After testing a variety of assays, the Hoechst nuclear stain was found to be convenient assays for quantifying cell proliferation. A screen of a chemical compound library using this Hoechst assay identified BSB660 as a novel inhibitor of VEGFR2. In the future, other chemical libraries will be tested using a TDA *in vitro* assay under development by other Blue Sky employees.

TABLE OF CONTENTS

Signature Page ……. 1

Abstract …… 2

Table of Contents ……..…… 3

Acknowledgements …….. 4

Background ……... 5

Project Purpose ……. 16

Methods …… 17

Results ……... 20

Discussion …… 25

Bibliography …… 27

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BACKGROUND

Angiogenesis

Angiogenesis is the process by which the body forms blood vessels. Angiogenesis is needed by the body during fetal development, the ovarian cycle, and tissue repair, and is also critical for advanced tumor formation. Angiogenesis can either create new blood vessels or repair damaged blood vessels. The process is comprised of several steps that result in the formation of functional blood vessels: cell proliferation, vascular permeability, cell migration, and cell survival as seen in **Figure-1** below (Holmes et al., 2007).

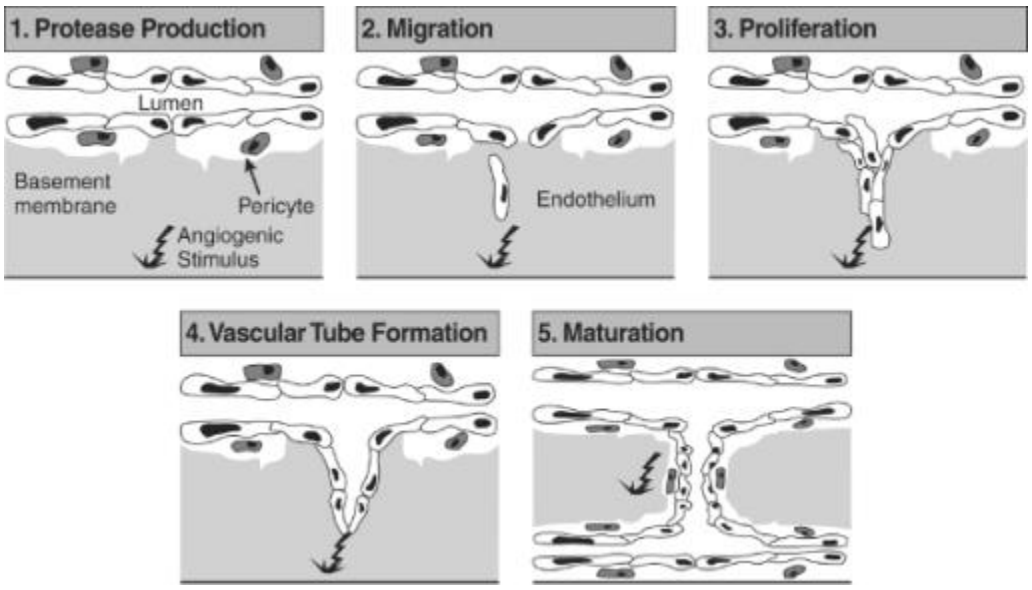

Figure-1: Diagram of the Stages of Angiogenesis. Box 1 shows the signal transduction of the cell to secrete proteases to allow cell migration. Box 2 shows the migration of the endothelial cell. Box 3 shows the proliferation and growth of the endothelial cell at the new site. Box 4 is the formation of the vessel by creating space between the two layers of cells. The final stage in box 5 is the maturation and survival of the vessel cells (Koch & Distler, 2007).

Permeability refers to the ability of substances to pass through the vessel's membrane. In most cases involving angiogenesis, only a selective exchange of materials occurs between blood cells and the endothelial cells lining the vessels. During angiogenesis, blood vessel endothelial

cells become more permeable to allow nutrients carried by the blood to reach more tissues throughout the body. When specific genes are over expressed in these endothelial cells, the cells secrete matrix-degrading proteases which breakdown the extra-cellular matrix holding the vessel together. This breakdown allows nutrients to pass through the wall of the blood vessel and enter the surrounding tissue (Dvorak et al, 1995).

With respect to cell migration, there are multiple different reasons cells migrate, but in relation to angiogenesis, endothelial cells migrate to new locations to generate new vessels or to repair old layers of blood vessels. Researchers have discovered three different mechanisms by which cells migrate: chemotaxis, haptotaxis, and mechanotaxis. Chemotaxis is controlled by growth factors that push cells toward a gradient of soluble chemo-attractants. Haptotaxis is cell migration towards a gradient of immobilized ligands, controlled by integrins binding to ECM components. Mechanotaxis is cell migration in response to physical forces placed on cells by fluids. In the case of angiogenesis, endothelial cell migration usually occurs by chemotaxis. Growth factors, such as VEGF secreted by injured or diseased cells, bind receptors on endothelial cells which respond to these signals and move towards the area in need of vascularization (Lamalice et al., 2007).

After endothelial cells have migrated to the needed area, they can grow and divide to produce blood vessels. Proliferation at the site increases the speed of angiogenesis; without it all the cells needed to create a vessel would need to migrate to the site. Once a few select endothelial cells have been placed in the correct spot, the cells can grow and divide to produce a blood vessel (Holmes et al., 2007).

The final step of angiogenesis is cell survival. It is crucial for angiogenesis that new endothelial cells do not die to prevent gaps in the blood vessels and fluid leaking (Holmes et al., 2007).

VEFG and VEGFR Role in Angiogenesis

In 1983, Paul Senger discovered a tumor-secreted vascular-permeability factor (VPR) that functioned to stimulate tumor angiogenesis (Ribatti, 2011). Six years later, Ferrara and Henzel isolated an endothelial cell-specific mitogen which they named vascular endothelial growth factor (VEGF) (Ferrara & Henzel, 1989). Later that same year, Daniel Connelly discovered that VPF and VEGF were in fact the same protein and it is crucial for angiogenesis (Ribatti, 2011).

In humans, the VEGF family is comprised of six different growth factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PLGF (placenta growth factor). These genes are only known to be expressed in tumor cells, macrophages, T cells, smooth muscle cells, kidney cells, mesangial cells, keratinocytes, astrocytes, and osteoblasts (Rosen, 2002). Expression of VEGF genes is normally only found during fetal development, the female reproductive cycle, and tissue growth and repair. The main regulator of the VEGF genes is oxygen tension. The genes contain a hypoxia response element (HRE) upstream, so when oxygen levels are low, transcription of the VEGF gene increases. If the body is not supplying enough oxygen to a specific location, the low oxygen triggers the expression of VEGF, and blood vessels form or become repaired (Neufeld et al., 1999).

VEGF needs to bind to a cell surface receptor for the growth factor to exert its effect. There are three known Vascular Endothelial Growth Factor Receptors (VEGFRs): VEGFR1,

VEGFR2, and VEGFR3. Receptors 1 and 2 are tyrosine kinases found in vascular endothelium cells (Holmes et al., 2007). VEGF-R2 and VEGF-R3 are found in lymphatic endothelium cells. VEGFRs are tyrosine kinase receptors (TKRs). TKRs are the second most common type of cell surface receptors. Each VEGF receptor favors a specific (or a few) ligand(s). Once the ligand binds to its receptor, the receptor becomes phosphorylated at Tyrosine 1059, Tyrosine 951, Tyrosine 996, Tyrosine 1175, or Tyrosine 1212, which begins a signal-transduction cascade. This cascade then controls the expression and activity of genes, such as RAS and FAK, which stimulate angiogenesis (Ribatti, 2011).

Over-Expression and Under-Expression of VEGF and VEGFR

Although VEGF is a crucial gene for normal growth and development, there must be a very fine balance of VEGF and VEGFR in cells. If there is an overexpression or under-expression of one component, it will lead to pathological angiogenesis, an excess or inadequate neovascularization which in turn can lead to disease or even death (Holmes et al., 2007).

One disease resulting from the over-expression of VEGF or VEGFR is cancer. If VEGF and/or its receptors are overexpressed, more blood vessels form, increasing the feed to the tumor, allowing it to grow at a higher rate. During this process, tumor blood vessel development is usually chaotic, and the permeability of the blood vessels increase, allowing tumor cells to move more freely and metastasize (Jain, 2002). Researchers conducted an experiment to test the tissue of primary breast cancer tumors. They found that the density of blood vessels and the amount of VEGF present was highly correlated. Breast cancer is only one of many types of cancers that VEGF is involved in (Toi et al., 1994).

Another disease that VEGF plays a role in is psoriasis. Psoriasis is a skin condition where skin becomes red and irritated. The disease also involves inflammation, angiogenesis, and vascular remodeling. In 2006, a study was conducted to see if VEGF played a role in psoriasis, and the researchers found that overexpression of VEGF, HIF-1α, and MMP-2, leads to the inflammation and angiogenesis seen in patients with psoriasis (Simonetti et al., 2006).

A third disease involving over-expression of VEGF is adult rheumatoid arthritis. Rheumatoid arthritis is an inflammation of the joints and their surrounding tissues. Studies have shown that VEGF is responsible for the rapid blood vessel formation in these tissues. The rapid development leads to inflammation which causes pain and joint stiffness (Malemud, 2007).

Alternatively, if VEGF or VEGFR are under-expressed, then new blood vessels cannot form and damaged vessels cannot be fixed. This prevents blood from reaching growing and developing areas. In most cases, this under-expression leads to death (Holmes et al., 2007). One experiment conducted on mice in 1996 showed that embryos died within 12 days following knockout of one of the VEGF alleles. The effects of the missing allele were seen throughout the body. The forebrain was underdeveloped, the heart was weaker and there was failure in the formation of veins to other tissues such as the nervous system (Holmes et al., 2007).

VEGF-A

Most VEGF studies have focused on VEGF-A, as it is crucial for survival. Even a loss of one VEGF-A allele (located on chromosome-6) can lead to death. Also, VEGF-A is found in multiple isoforms, each isoform localizes to a different cellular location. For example, VEGF-A_{206} binds to the extracellular matrix, while a smaller isoform VEGF-A_{121} diffuse freely. These isoforms are made by alterative splicing of the 8 exons that make up the VEGF-A gene. VEGF-

A_{165} is the most abundant isoform, whereas VEGF-A_{145} and VEGF-A_{206} are rare. **Figure-2A** shows that the VEGF gene is comprised of 8 exons. Alternative splicing of these 8 exons creates the eight isoforms of VEGF-A shown in **Figure-2B**. Also seen in **Figure-2A** are other important domains of the VEGF gene including the dimerization site (that link to other VEGF molecules), receptor binding sites, a plasmin cleavage site (that activates plasmin that facilitates angiogenesis), a heparin-binding site (that controls the inflammatory response) and neuropilin-binding site (that controls cellular apoptosis) (Nowak et al., 2008).

Researchers have just begun researching the functions of the various VEGF-A isoform. Researchers have discovered anti-angiogenic isoforms, shown on the right in Figure-1B. Exon 8 has two subparts, 8a and 8b. If 8b is removed during splicing, an active angiogenic isoform will form, but if 8a is removed, an anti-angiogenic isoform will form. These anti-angiogenic isoforms act as competitive inhibitors, binding to the same active site on VEGFR as the angiogenic isoform, but rendering the receptor inactive (Nowak et al., 2008).

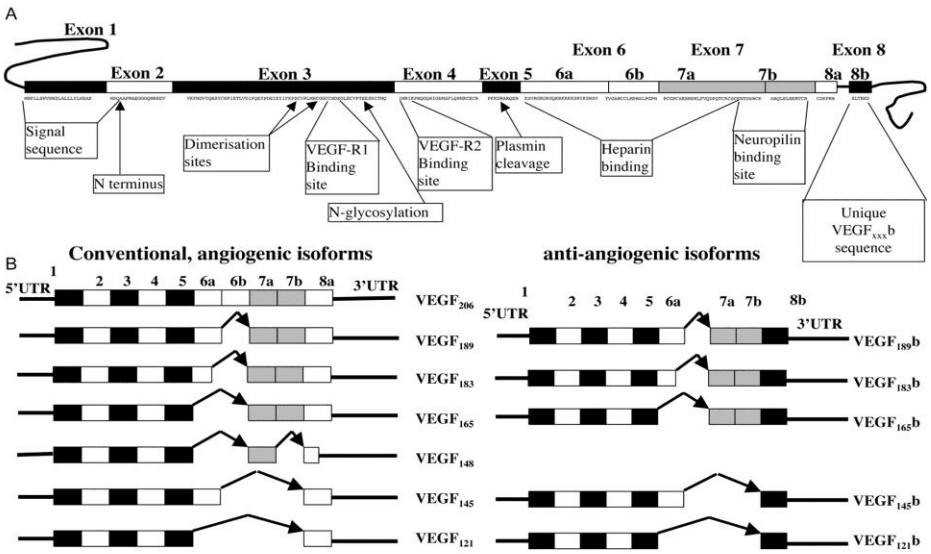

Figure-2: The VEGF-A Gene and Its Isoforms. Panel-A shows a model of the VEGF-A gene and its different domains. **Panel-B** shows the multiple different isoforms of VEGFA, both angiogenesis and anti-angiogenesis forms (Nowak et al., 2008).

VEGFR2

The receptor for VEGF-A, the best characterized VEGF and the one most crucial for survival, is VEGFR2. VEGFR2 is a 1356 amino acid tyrosine kinase receptor located on chromosome-4 (**Figure-3**). Seven immunoglobulin domains make up the extracellular portion of the receptor. Different ligands bind to different surface domains, but VEGF-A binds to domains II and III. The intracellular portion of the receptor consists of two kinase domains (Holmes et al., 2007).

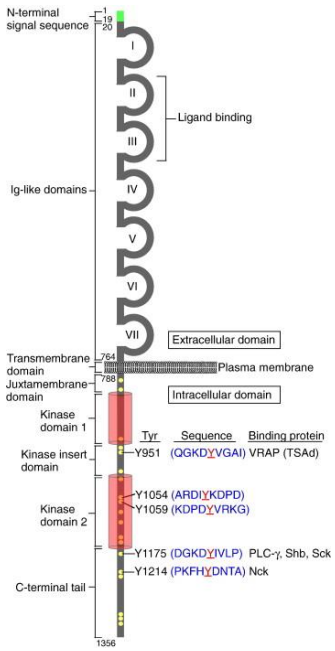

Figure-3: Diagram of the Structure of VEGFR2. The extracellular portion of the receptor contains seven Ig-like domains, two of which bind ligand. The intracellular portion of the receptor contains two kinase domains (red) (Holmes et al., 2007).

Once a ligand is bound to VEGFR2, the receptor dimerizes, and then it autophosphorylates. The receptor contains multiple tyrosine phosphorylation sites, which leads to activation of five different major pathways (**Figure-4**) (Ivy et al., 2009). The Ras pathway (diagram left) is a series of proteins that become active with phosphorylation. Once one protein in the series becomes active, it will phosphorylate the next protein, culminating with the

activation of ERK which induces the transcription of genes related to cell proliferation and migration. Alternatively, activation of VEGFR2 by phosphorylation at site pY1175 on its SH2 domain activates PLC-γ (diagram second column) (Ivy et al., 2009). PIP2 is cleaved into second messengers DAG and IP3. DAG then activates PKC, while IP3 causes a release of intracellular calcium from the endoplasmic reticulum. The release of calcium and the activation of PKC results in the induction of eNOS, cPLA and NO, which in turn controls endothelial cell permeability (Cross et al., 2003). Activation of the focal adhesion kinase (FAK) pathway and p38 (diagram third and fourth columns) lead to cell migration. FAK activates paxillin, while p38 induces heat shock protein-27, which is followed by actin reorganization and cell migration. The PI3K pathway (diagram right side), controls cell survival. PIP2 is converted to PIP3 by the PI3K catalyst, which activates Akt. This kinase inhibits two pro-apoptotic proteins (caspase-9 and BAD) to increase cell survival (Ivy et al., 2009).

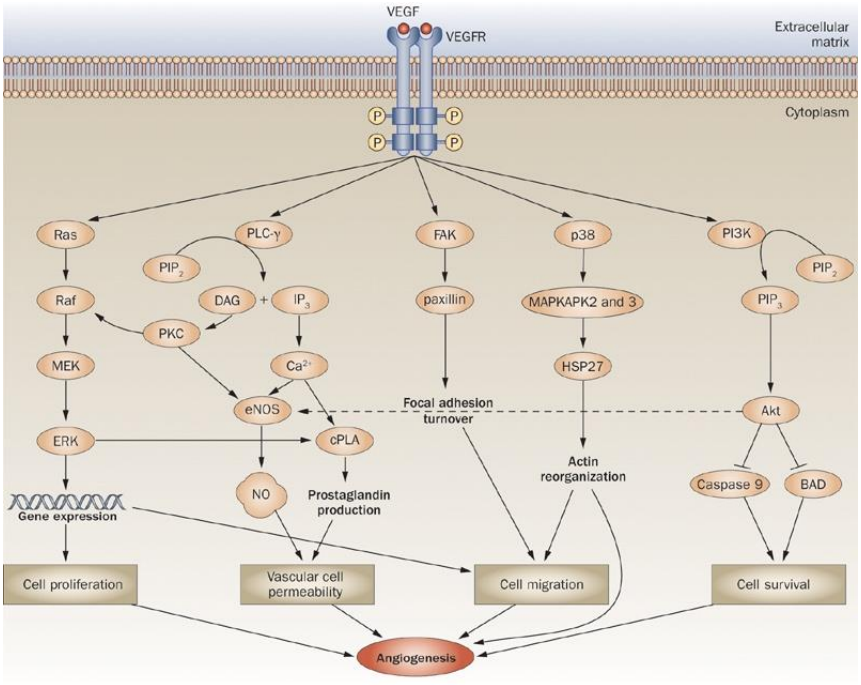

Figure-4: Diagram of Various VEGF Signaling Pathways. Shown are five major pathways downstream from VEGFR2 dimerization. (Ivy et al., 2009)

VEGF and VEGFR Inhibition

Because the activation of VEGF and its receptor has been linked to several disorders (cancer, arthritis, psoriasis, etc.) researchers have focused on identifying methods for inhibiting their activation (**Figure-5**). These methods include using VEGF monoclonal antibodies to bind to receptors, coupling toxins to VEGF (to kill cells bound to the ligand), using soluble VEGF receptors as competitors to bind free VEGF, using peptides that interfere with VEGF binding its receptor, and using agents that block VEGFR signaling (Rosen, 2002). Unlike other cancer treatments, VEGF inhibitors do not directly kill the tumor cells, but act to block tumor angiogenesis.

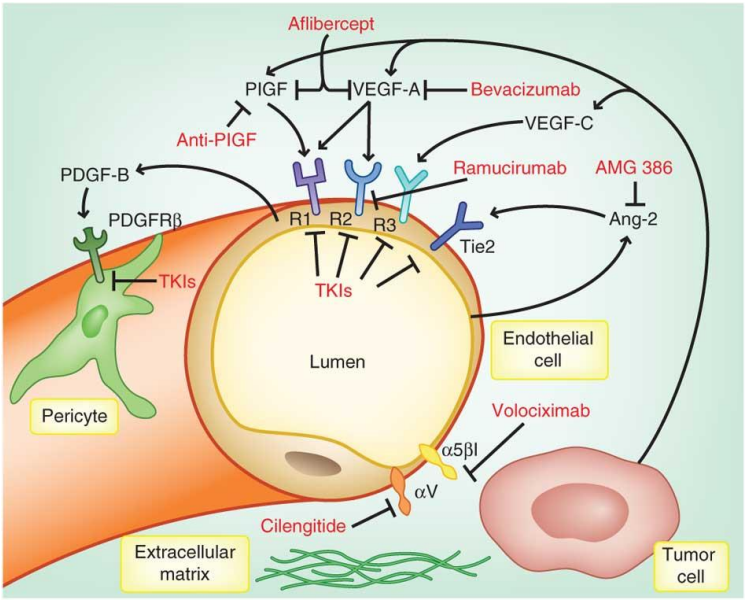

Figure-5: Diagram of Methods Used to Inhibit VEGF. The figure shows different modes of VEGF inhibition and different known inhibitors. The figure shows two major types of inhibition: monoclonal antibody inhibitors and tyrosine kinase inhibitors. The two forms of monoclonal antibodies are those that target growth factors, such as bevacizumab and those that target receptors, such as ramucirumab. The tyrosine kinases inhibitors can be found within the lumen and block the receptor signals (Singh and Ferrara, 2012).

VEGF monoclonal antibodies attach to the VEGF ligand to block its function. In 1997, researchers were able to humanize an anti-VEGF IgG, bevacizumab, produced in mice for

delivery to cancer patients. This antibody inactivates all human VEGF-A isoforms. After bevacizumab was humanized, researchers began testing its effects on colorectal cancer, metastatic breast cancer, non-small-cell lung cancer, renal cell carcinoma, hormone-refractory prostate cancer, gastrointestinal stromal tumor patients (Ferrara et al, 2007). In many cases, the bevacizumab inhibited the growth and spread of tumors, and when used in parallel with chemotherapy the combination was especially effective. The FDA has approved bevacizumab (known as Avastin on the market) as a first-line cancer treatment in combination with chemotherapy for colorectal cancer (Singh and Ferrara, 2012). More recently, the FDA has also approved bevacizumab for treating non-small cell lung cancer, metastatic kidney cancer, and glioblastoma (Genentech USA, Inc, 2013).

Another form of VEGF inhibition is competitive inhibition. Researchers have found different molecules that can bind to the VEGFRs and inhibit the receptor's activity. One promising drug that functions as a VEGFR inhibitor is Axitinib, which stabilizes the kinase domain of the VEGFRs and blocks signal transduction (Kelly and Rixe, 2009).

Unfortunately, inhibiting VEGF can create complications in a patient. In many cases, the inhibition is not permanent. This could mean that medicine must continuously be taken to prevent rapid angiogenesis, or if medicine is only given one-time the disease can return. This is especially true for cancer. Also, angiogenesis and VEGF are still being researched, and new harmful side effects will likely be discovered. In addition, researchers worry that the inhibition of VEGF and VEGFR might lead to the up-regulation of other growth factors to accomplish the cell proliferation and migration (Rosen, 2002).

Blue Sky Biotech and Project Purpose

Located in Worcester, MA, Blue Sky Biotech is a Contract Research Organization who prides themselves in high quality research. Blue Sky offers services in different areas including molecular biology, cell culture, protein sciences and assay services. The staff is capable of handling projects from basic to more complex and customized projects for clients. The departments work closely together to insure speed and quality of their services. Everyday tasks range from gene synthesis to protein purification to assay development.

PROJECT PURPOSE

The goal of this project is to develop an *in vivo* high through-put screening assay to identity VEGFR inhibitors as potential blockers of angiogenesis. If such an assay can be developed, it could be offered to clients as a cheaper and quicker method of finding inhibitors of specific receptors. Potential drugs will be evaluated *in vivo* and later *in vitro* by Blue Sky employees, in hopes that compounds that were hits *in vivo* will also be hits *in vitro*. If correlations are found between *in vitro* and *in vivo* activities, it would allow Blue Sky to analyze large libraries of drugs quickly and relatively inexpensively *in vitro*, and then test potential positives *in vivo*.

The cell culture-based "*in vivo"* assay utilizes human umbilical vein endothelial cells (HUVECs) and the Hoechst nuclear stain as a convenient method for assaying cell proliferation, an indicator of VEGFR-pathway activation or inhibition. A long-term goal of Blue Sky Biotech is to develop this comparative assay with an additional goal of identify novel inhibitors of angiogenesis that could potentially be used to treat cancer, psoriasis, and arthritis.

METHODS

Cell Culture and Maintenance

The Human Umbilical Vein Endothelial Cells (HUVECs) were pooled samples obtained from Lonza Biologics Inc (Hopkinton, MA). The HUVECs were grown in EGM-MV obtained from Lonza (Catalog #3125). The EGM-MV is made up of EBM basal media and the MV BulletKit containing Bovine Brain Extract with heparin, hEGF, hydrocortisone, Gentaminicin Amphotericin B and Fetal Bovine Serum.

The cells were passaged every 2-4 days or until about 80% confluence was reached. The medium was removed from the cells, and then the cells were washed with 1X PBS. Once washed, 0.05% trypsin was used to detach the cells. When cells were detached, new medium was added to the cells and the cells were placed in a new flask seeded at 15,000-20,000cells/cm^2.

Cell Counts

The cell count was used as a preliminary test of the effect of VEGF and its known or potential inhibitors on cell proliferation. The cells were plated in 96-well plates in EGM-MV media, at a density of 5,000 cells/well. The cells were incubated for 30 hours at 37˚. After 30 hours, the medium was changed. The plate was washed three times with 200ul of 1X PBS each time. Cells were then left in either EGM-MV or starvation serum (EBM basal media + 0.2% FBS) for 18 hours. After 18 hours in the new medium, VEGF and the drugs were added to the cells. Finally, after 24 hours the cells were fixed, stained (with a nuclear Hoechst stain, procedure below) and imaged.

Nuclear Stain

The wells were washed three times with 200 ul of 1X PBS. The cells were fixed for 10 minutes using 50ul 4% paraformaldehyde. Once again, the cells were washed three times with 200 ul of 1X PBS. A Hoechst stain obtained from Invitrogen (Catalog #H3569) was added to the cells (50 ul/well) for 10 minutes. Finally, the cells were washed three more times with 1X PBS and then filled with 200 ul of the 1X PBS. The plate was then imaged using a Cellomics Cellinsight High Content Screening Platform.

Click-iT® EdU Staining

The Click-iT® EdU kit was obtained from Invitrogen (Click-iT® EdU Alexa Fluor® 488 HCS Assay – C10350), and the protocol was adjusted slightly for these experiments. 90 minutes prior to staining, 50 ul of a 2X working solution of EdU was added to the cells. The cells were incubated with the EdU for two hours. After the two hours, the cells were fixed using 3.7% paraformaldehyde. 50 ul of the paraformaldehyde was added to each well and left at room temperature on a slow rocker for 15 minutes. The paraformaldehyde was then removed, and the wells were washed twice with 200 ul of PBS. 50 ul of a permeabilization/block solution (96.9% PBS, 3% FBS, 0.1% Triton X-100) was then added to each well, and the plate was again left at room temperature on a slow rocker for 15 minutes. The block solution was removed and the wells were washed again 2 times with 200 ul of PBS. Next, 50 ul of Click-iT® EdU Reaction Cocktail (85.5% 1X Click-iT® EdU Reaction Buffer, 4% $CuSO_4$, 0.25% Alexa Fluor 488®, 10% 1X Click-iT® EdU Buffer Additive) was added to each well, the plate was covered in aluminum foil (to protect the stain from the light), and placed at room temperature, on a rocker, for 30 minutes. The reaction cocktail was removed, and the wells were washed once with 50 ul

Click-iT® Reaction Rinse buffer. Next, 100 ul of HCS Nuclear Mask Blue (1:2000 dilution in PBS) was added to each well and gently rocked at room temperature for 30 minutes. After the 30 minutes, the wells were washed twice with 200 ul of PBS, the wells were filled with 200 ul and then covered with aluminum seal film. The cells were then imaged using the Cellomics Cellnsight High Content Screening Platform.

RESULTS

A long term goal of Blue Sky Biotech is to develop an assay that can identify novel inhibitors of angiogenesis that can potentially be used to treat cancer, psoriasis, and arthritis. This MQP project aims to contribute to this effort by designing a rapid cell culture-based assay for inhibitors of vascular endothelial growth factor receptor-2 (VEGFR2) utilizing human umbilical vein endothelial cells (HUVECs). Since activation of the VEGFR2 pathway in angiogenesis leads to cell proliferation, the proliferation of HUVECs (or their inhibition) will be monitored by assaying a classic cell proliferation marker Ki67 in the presence or absence of potential VEGFR2 inhibitors from a library of compounds available at Blue Sky Biotech.

The project was initiated by treating HUVEC cells with VEGF and known VEGFR2 inhibitors, and then assaying the cellular levels of protein Ki67 proliferation marker using immunofluorescence with an antibody against Ki67 (primary antibody: Mouse anti-human Ki67, secondary antibody: Alexa Fluor goat anti-mouse IgG) and Hoechst stain. The cells were grown in EGM-MV basal medium for 24 hours, after which known inhibitors and VEGF were added. After a second 24-hour incubation, the cells were fixed, stained with Alexa Fluor 488 (Ki67 stain) and Hoechst dye (nuclei stain), and imaged. Sample images are shown in **Figure-6**. **Figure-6A** represents one field of view from the untreated wells (EGM-MV basal medium alone). **6B** represents a field of view from the positive control (EGM-MV+VEGF). The Ki67-positive cells are shown in the second column, and as the bright areas in the third column (overlay). As seen in the images, there is a higher number of cells in the positive control VEGF-treated cells compared to those treated with basal medium, however there appears to be no significant difference between the percent Ki67-positive cells with and without VEGF-A.

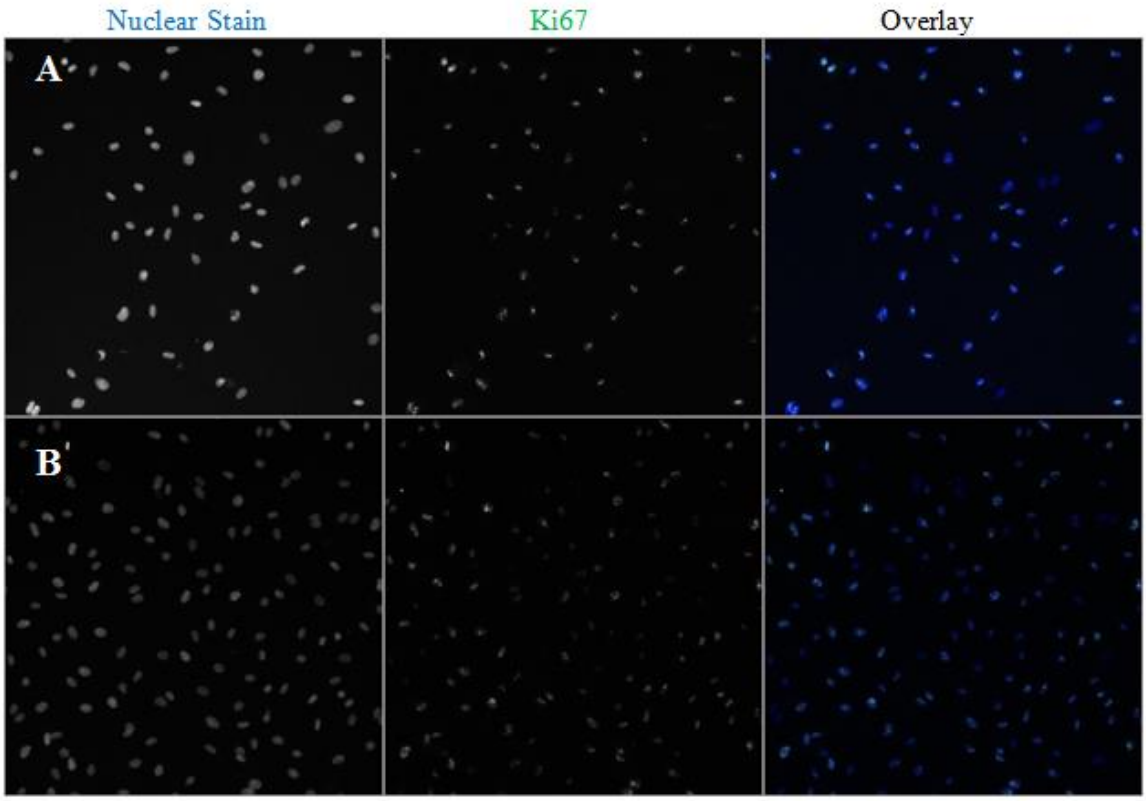

Figure-6: Experiment-1: Ki67 Staining Results of HUVEC Cells Treated With and Without VEGF. Images were obtained from the imaging software of one field of view from one well in basal medium alone (Panel-A) and medium + VEGF (Panel-B). The first column represents the nuclear stain showing all of the cells in the field of view. The second column denotes the Ki67-positive cells in the G_1, S or G_2 phase of the cell cycle. The right column shows the overlay of the two images.

Cell counts were not quantitated in the first experiment, so a second experiment was initiated with this goal. Cells were plated and allowed to grow for 48 hours, and then known inhibitors and VEGF were added to the appropriate wells. After incubating an additional 48 hours, the cells were fixed, stained with Hoechst nuclear stain, and imaged (**Figure-7**). **Figure-7B** shows an image of the positive control (cells treated with VEGF without inhibitor). Comparing this image to cells treated with medium only (7A), or those treated with VEGF plus inhibitors, it is clear that as expected there are more cells in 7B than any other condition.

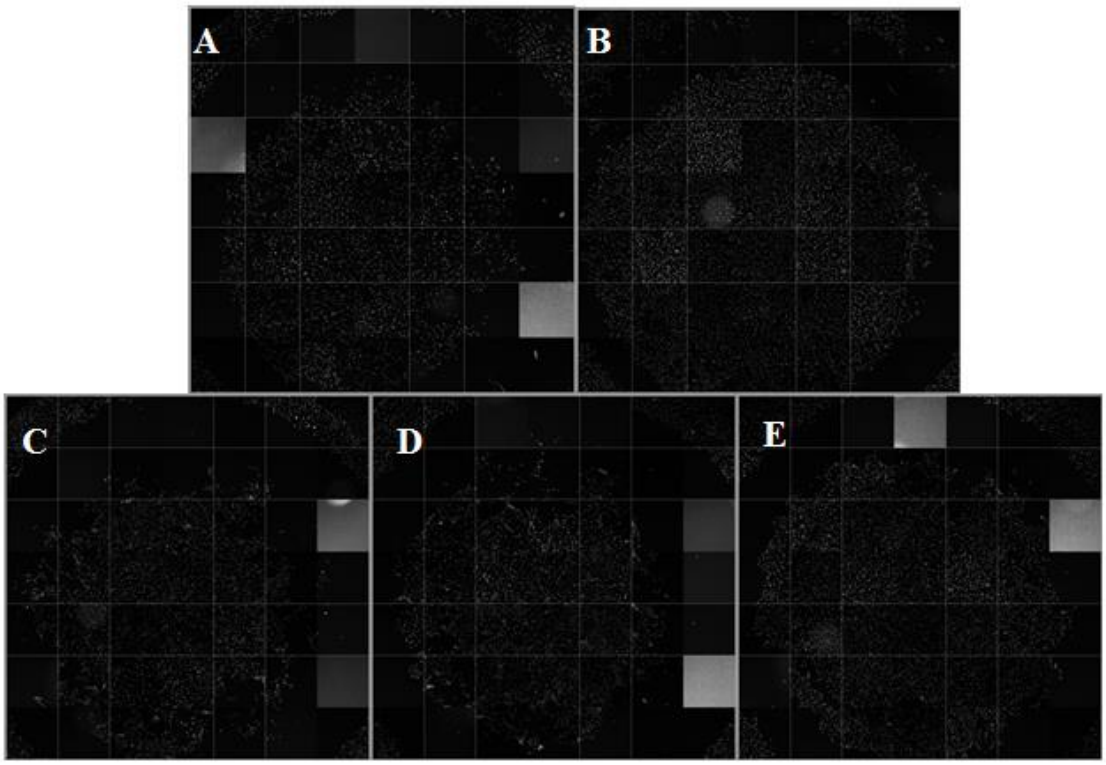

Figure-7: Experiment-2: Images of HUVEC Cells Treated with VEGF or Various Known VEGF Inhibitors and Stained with Hoechst Nuclear Stain for Purposes of Obtaining Cell Counts. The images denote 49 fields (small white boxes) of view from one well in each condition. Panel-A represents control cells (medium alone); B is cells treated with VEGF; C is treated with VEGF plus Axitinib VEGF-inhibitor; D is cells treated with VEGF plus VEGF-inhibitor Ki8751; E is cells treated with VEGF and VEGF-inhibitor ZM323881. All inhibitors were tested at 100 nM.

The results of experiment-2 were quantified from the images, and then analyzed using ANOVA (**Table-I**). VEGF (histobar-2) caused a significant 2-fold increase in cell numbers relative to basal medium (histobar-1). The vehicle DMSO (used to solubilize the inhibitors) caused no measurable decrease in cell number (histobar-3). Each of the three VEGF-inhibitors tested (Axitinib, histobar-4; Ki8751, histobar-5; ZM323881, histobar-6) decreased cell numbers back to the control level. The inhibited cells appeared normal, and did not appear to be killed by the drug.

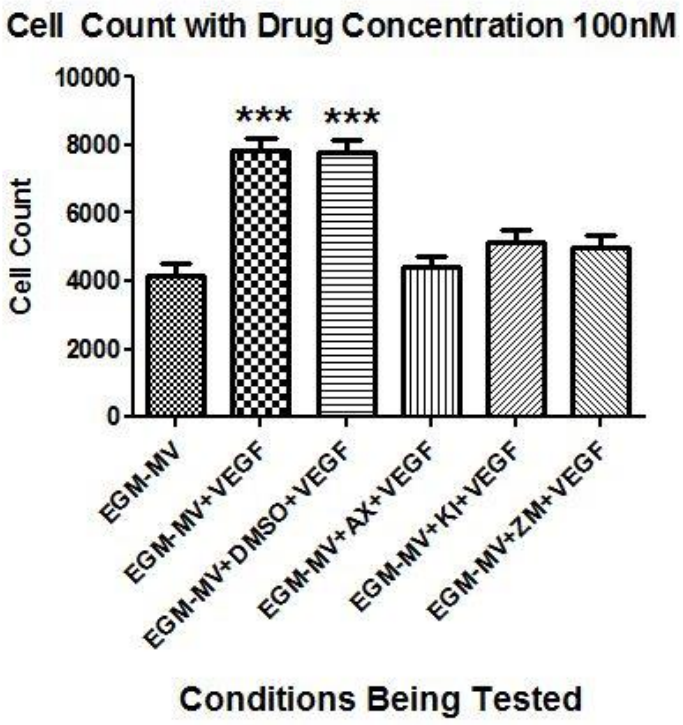

Table-1: Quantitation of Cell Numbers from the Hoechst Nuclear Stained Microscopy of the Previous Figure.

Once it was confirmed that VEGF stimulated cell division, and the known inhibitors blocked the VEGF-stimulation, a different stain, EdU, was used to measure the quantity of BrdU incorporated into DNA, another measure of cell proliferation. The cells were plated on day-1 with (or without) VEGF. Axitinib and novel inhibitors were added on day- 3 (48 hours after plating), and on day-5 the cells were fixed and stained (48 hours after treatment). In this procedure, EdU was added to the cells 90 minutes prior to fixing and staining. The EdU readouts were inconclusive, but the cells were stained with Hoechst dye for cell count determinations (**Table 2**). Twenty four different chemical compounds from Blue Sky Biotech were tested against the negative control with medium alone (histogram-1). The cells treated with VEGF

(histogram-2) and or VEGF and DMSO (histogram-3) were significantly higher than the control. Cells treated with Axitinib and VEGF (histogram-4), showed a slightly reduced cell count, although this known inhibitor did not inhibit the VEGF response as much as it had in previous experiments. However, three other novel compounds seemed to be potential hits: BSB655, BSB660, and BSB6, which showed reduced cell number statistically equal to the control ($p < 0.001$).

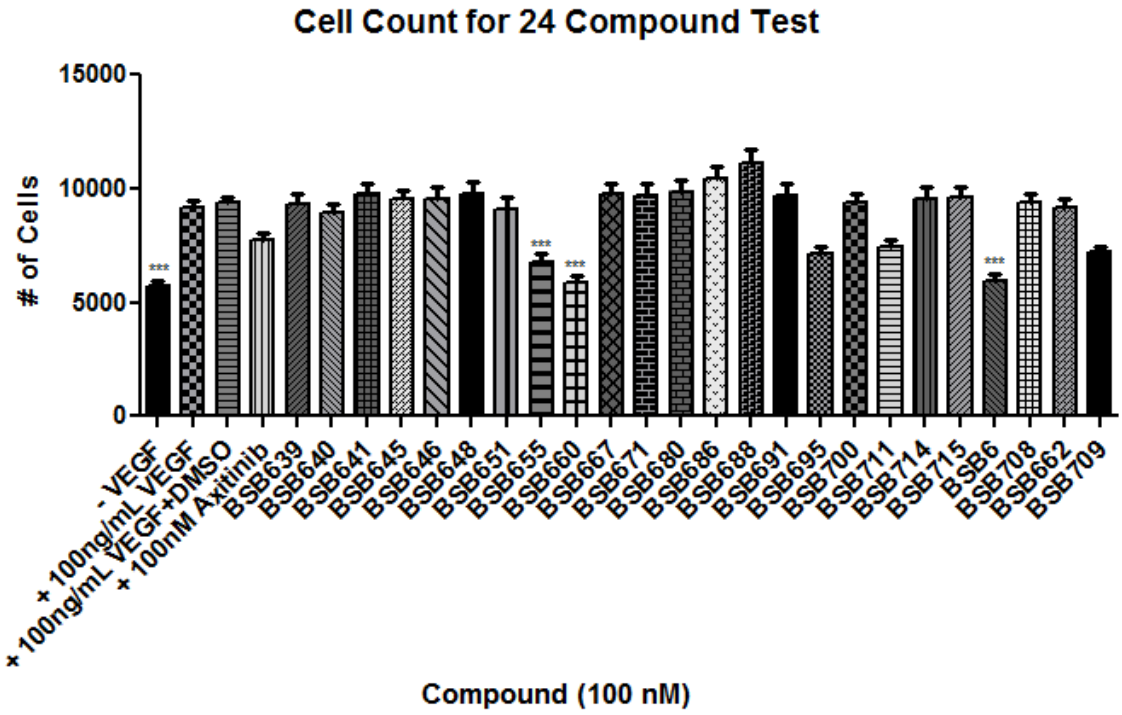

Table-2: Quantitation of Hoechst Nuclear Stained Cells from Experiment-3.

The results of the cell culture based "*in vivo"* assay were then compared to the TDA *in vitro* assay developed by Blue Sky employees (data not shown). This biochemical assay shared one similar positive hit with the *in vivo* results shown above, compound BSB660.

DISCUSSION

The major conclusions drawn from this project were that a Hoechst nuclear stain can conveniently be used to quantify HUVEC cell proliferation in a cell culture based "*in vivo"* assay to identify potential VEGFR inhibitors, and can successfully be used to screen a library of chemical compounds for potential VEGFR inhibitors.

In the Hoechst nuclear stain assay, VEGF-A_{165} increased the proliferation of HUVECs at least 2 fold, and a known VEGF-inhibitor Axitinib almost completely inhibited proliferation in the present of VEGF. The known VEGF inhibitor Ki8751 did not work well in the Hoechst nuclear stain assay, so perhaps is not as strong an inhibitor as previously thought (Kelly & Rixe, 2008). It was also shown that the known inhibitors Axitinib, Ki8751, and ZM323881, do not appear to be cytotoxic, but rather inhibit cell proliferation and growth, as previously thought (Rosen, 2002).

In the 24 compound Hoechst and BrdU assay, the screening of Blue Sky's chemical compound library identified three potential VEFGR inhibitors (BSB655, BSB660, and BSB6) that kept the cell count in the presence of VEGF at the control levels. One of those compounds, BSB660 was subsequently verified to have VEGFR inhibition activity in Blue Sky's TDA *in vitro* screening assay.

Many problems arose during the development of the assay and screening of chemical compounds. The first issue was with the Ki67 proliferation marker staining procedure. Ki67 is supposed to stain any cell in the G1, S, or G2 phases of the cell cycle. Unfortunately, HUVEC cells spend most of their time in one of these three cell cycle phases, so the Ki67 stain was too broad for the purposes of this assay. After searching for a new stain, EdU was found. EdU is

incorporated into DNA during synthesis (S phase), which narrowed down the positive cells. The dye binds cells in S phase at the time of exposure, which was two hours prior to staining in this project. The problem with this EdU procedure was it eliminated all cells that had previously passed through S phase, which had potentially proliferated in response to VEGF. The other issue with EdU is that once it has been incorporated into the cell, the cell cannot divide any further, which reduced the accuracy of the Hoechst nuclear cell count if performed at the same time (Table 2).

In the future, the next step would involve using the Hoechst nuclear stain assay to screen more chemical compounds to identify other novel VEGFR inhibitors, and then comparing the results to the TDA *in vitro* assay and running a Western blot on potential positive hits to determine whether the potential inhibitor reduced expression of a gene known to be stimulated by VEGF. Showing that similar compounds score as positives in both the *in vitro* and *in vivo* assays would allow Blue Sky Biotech to offer these combined techniques as a new and affordable assay for clients looking to screen large libraries of compounds, while saving clients and Blue Sky time and money, leading to quicker, more accurate results. Finally, compound BSB660 can be researched further as a novel inhibitor of VEGFR to develop new drugs for treating VEGF-related cancers, psoriasis or arthritis.

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