

Abstract

Angiogenesis is the formation of new blood vessels and has been implicated in multiple human diseases including cancer, diabetic retinopathy, and rheumatoid arthritis (Carmeliet, *Oncology* 69:4-10, 2005). Endothelial cell proliferation is known to occur in early stages of angiogenesis (Coutas et al., *Nature* 438:937-45). As such, the identification of compounds that selectively inhibit endothelial cell proliferation represents an attractive strategy for the development of novel drugs, which act by inhibiting angiogenesis. In a high throughput screening campaign two human cell lines, primary human umbilical vein endothelial cells (HUVEC) and human lung fibroblasts (LL47) were used to identify compounds that selectively inhibited human endothelial cells. It is anticipated that compounds showing specific activity in the endothelial cells will provide useful research tools for angiogenesis research and potential leads for anticancer drug discovery. An overview of the HTS campaign, including the validation experiments and the results from the screening of a 100,000 compound library from the NIH Small Molecule Repository will be presented.

Introduction

The formation of new blood vessels, termed angiogenesis, is a multi-step process that includes endothelial cell activation, proliferation, migration and differentiation (fig 1). The differentiation includes capillary tube formation with a lumen leading to new blood vessel formation. This process is essential for tissue and organ growth during development and repair after injury. However, in healthy adult humans blood vessels remain mostly stable and endothelial cells remain primarily inactivated. The process of endothelial cell activation is tightly regulated by a balance between angiogenic and anti-angiogenic molecules in the human body. The disruption of this balance and subsequent uncontrolled blood vessel formation has been implicated in pathologies including cancer, arthritis and diabetic retinopathy, among others¹. This relationship between endothelial cell activation and pathogenic angiogenesis makes the pharmacological inhibition of endothelial cell activation and subsequent growth an attractive target for drug discovery. Further, because small blood vessels such as those found within growing tumors and those associated with vascular disease are made up entirely of endothelial cells, this cell type is perhaps a more attractive target than pericytes or smooth muscle cells found in larger vessels.

The most well known compounds that inhibit endothelial cell growth target vascular endothelial growth factor (VEGF). VEGF is a sub family of growth factors that are important signaling proteins involved in both vasculogenesis and angiogenesis that has been shown to be overexpressed in most human solid tumors². Although several compounds targeting VEGF have entered advanced clinical trials, resistance to anti-angiogenic compounds targeting only this family of growth factors has recently emerged. It is assumed that this is related to other growth factor mediated signaling pathways. Because of this resistance, it is important to develop molecular probes that target other growth factors or proteins, such as FGF and PDGF, involved in endothelial cell activation to help elucidate the mechanisms of angiogenesis.

The process of finding new chemical probes for this pathway began with the development of an HTS assay utilizing two different cell lines. Human umbilical vein endothelial cells (HUVEC) were grown in the presence of test compounds to assess the compounds' ability to inhibit endothelial cell growth. To test the specificity of the test compounds for endothelial cell inhibition, a counter screen was performed using a human adult pulmonary fibroblast cell line (LL47). Those compounds that demonstrated differential inhibitory activity against the HUVEC line when compared to the LL47 cell line were identified as specific hits. These specific hits were then tested in dose response. The compounds that confirmed in dose response were then tested in a number of secondary assays, including in vitro endothelial cell migration and tube-formation assay, and in vivo CAM, Xenograft CAM and mouse Matrigel Plug assay.

Materials and Methods

Compounds

This assay was screened against the Molecular Libraries Small Molecule Repository (MLSMR) library. The primary single dose screen was screened against the entire MLSM library which consisted of approximately 86,000 compounds at the time of the screen. All compounds were solubilized in 100% DMSO at a concentration of 10mM. A concentration of 0.1% DMSO was maintained in all test and control wells in the assay plates. The positive control used in the assay was 1µg/mL MEPR-X, a toxic nucleoside analog.

Cell Lines

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA). These primary cells were maintained in Endothelial Cell Basal Medium (EBM) supplemented with 2% fetal bovine serum, 12 µg/mL bovine brain extract, 1 µg/mL hydrocortisone, and 1 µg/mL GA-1000 (gentamicin-amphotericin). HUVEC cells were maintained no more than twelve passages. The culture media was also used as assay media.

Human lung fibroblasts (LL47) were obtained from the ATCC. These cells were grown in Improved MEM (Richter's modification) media supplemented with 15% fetal bovine serum and 50 µg/mL gentamicin. LL47 cells were maintained no more than twelve passages. The culture media was also used as the assay media.

Endothelial Cell Growth Inhibition Assay

HUVEC cells were plated at a density of 1250 cells / well in 20µL into collagen coated black clear bottom tissue culture treated 384 well assay plates. The cells were dispensed with a Matrix WellMate non contact dispenser. Assay plates were incubated 1h at room temperature before being moved to incubators maintained at 37°C, 5% CO₂. After a 24h incubation, test compounds and controls were diluted in assay media to a final test concentration of 10 µM. Assay plates were returned to the incubator for 72h. At the end of this incubation period, assay plates were removed from the incubator and equilibrated to room temperature for 30min. Once equilibrated, cell viability was read by adding 25 µL of Cell-Titer Glow (Promega Inc) to each well and reading the luminescent readout on a PerkinElmer Envision.

LL47 Fibroblast Counter Screen Assay

The assay conditions for the counter screen were identical with a few exceptions. The cell line and assay media were switched to the LL47 cells and media. The assay plates used were not collagen coated.

High Throughput and Dose Response Screens

The first screen was a 10,000 compound screen screened in duplicate to verify reproducibility of the assay. After validation, the primary screen of 86,000 compounds was run at 10µM in single dose. Both the endothelial cell inhibition assay and the counter screen were run against the full library. Compounds that demonstrated >70% inhibition of HUVEC cells and <30% inhibition of LL47 line were identified for dose response follow up. The hits from the primary screen were then tested in 10 point dose response ranging from 0.039 µM to 20 µM. Compounds that confirmed as showing the best differential activities when comparing both assays were identified for structure activity analysis and secondary assay follow up.

Secondary Assays

Endothelial Cell Migration Assay: endothelial cell migration is one of key steps of the angiogenesis process, which is crucial for on-site recruitment of blood vessel formation. The trans-well filter/inserts chamber system is used for endothelial cell migration assay.

Endothelial Tube Formation Assay is based on the ability of endothelial cells to differentiate into capillary-like tubes when cultured on a gel of basement membrane extracts, such as Matrigel. This assay represent a simple but powerful model for studying inhibition and induction of angiogenesis.

Chick embryo CAM assay is an *in vivo* new blood vessel formation assay. Antiangiogenic activity of a testing agent can be quantitatively analyzed with CAM assay.

Xenograft CAM assay: human tumor cells are implanted on top of CAM. This assay is used to evaluate compound effect on both new blood vessel formation and tumor growth.

Mouse Matrigel Plug assay is an *in vivo* new blood vessel formation assay. CD31 immunostaining is used in quantizing antiangiogenic activity of a testing agent.

Figures

Figure 1. Angiogenesis

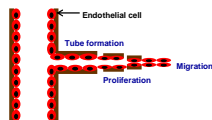


Figure 3. Spearman's Correlation (0.69)

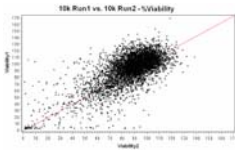


Figure 5. Dose Response to HUVEC cells

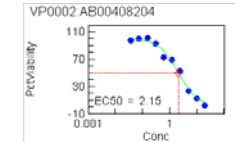


Figure 7. Endothelium Cell Migration Assay

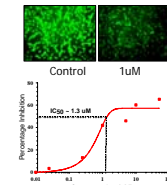


Figure 2. VEGF Assisted Tumor Growth

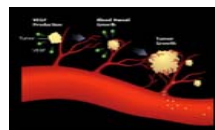


Figure 4. Z' Values for 10k Screen

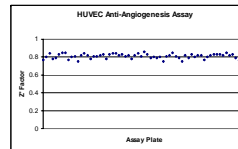


Figure 6. Dose Response to LL47 cells

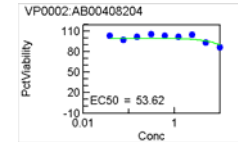
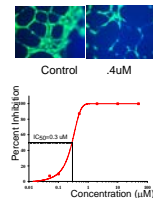


Figure 8. Endothelial Tube Formation Assay

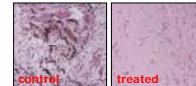


Figures

Figure 9. Chick Embryo CAM Assay



Figure 10. Mouse Matrigel Plug Assay



Results

After development and implementation of both the anti-angiogenesis assay using HUVEC cells and the counter screen using LL47 fibroblast cells, a 10,000 compound diversity set was run in duplicate to measure the reproducibility and robustness of the proposed HTS campaign. When the two 10,000 compound validation screens were compared, the assay showed high reproducibility with a Spearman's coefficient of 0.69 (fig. 3) and excellent robustness with Z' factors averaging nearly 0.8 (fig. 4).

The primary single dose screen was run in both assays against the entire MLSM library available at the time, consisting of approximately 86,000 compounds. In the anti-angiogenesis screen, 925 compounds demonstrated cell viability in HUVEC cells of <30% and were named actives (PubChem AID: 648). In the LL47 counter-screen, 937 compounds demonstrated cell viability of <50% and were considered actives (PubChem AID: 719). The active compounds from each screen were tested in dose response and 576 compounds confirmed as active in the primary screen (PubChem AID:822) and 314 compounds were confirmed as active in the counter screen (PubChem AID:821). By comparing IC50 values of the active compounds, specificity for HUVEC cells was analyzed. Those compounds that demonstrated calculated IC50 values in the HUVEC assay, but showed no activity (IC50 > High dose) in the LL47 counter screen were identified as HUVEC specific. This was a total of 289 compounds. An example of dose response curves to both cell lines can be seen in figures 5 and 6.

The 289 compounds identified as HUVEC specific underwent structure activity analysis and a number of chemical scaffolds were identified and pursued for optimization by the Medicinal Chemistry group. Using these chemical scaffolds a number of lead compounds have been synthesized and tested in secondary assays. These assays include the endothelial cell migration assay (fig. 7), the endothelial tube formation assay (fig. 8), the chick embryo CAM assay (fig. 9) and the mouse Matrigel plug assay (fig. 10).

Work is continuing on this project. Currently several very potent compounds are being pursued and the group is excited about the results especially in the medicinal chemistry and secondary assay areas. As this project moves forward the community can expect to be kept abreast of future findings through the NCBI PubChem Project.

Acknowledgments

This work was funded by NIH Molecular Libraries Screening Center Network, Contract 1 U54 HG003917-002.

References

- Carmeliet, P. Angiogenesis in health and disease. *Nat. Med.* 9, 653-660 (2003).
- Gariano, R. F. & Gardner, T. W. Retinal angiogenesis in development and disease. *Nature* 438, 960-966 (2005).