Introduction

Cellular kinases play an important role in relaying signals from activated receptors residing at the cell membrane to the interior of the cell through signal transduction. The cellular processes in which they are involved include angiogenesis, cell growth, cell migration, and apoptosis. Overexpression of the kinase, or constitutive activity, has also been linked to a number of disease states, including vascular disease, bone disorders, and multiple forms of cancer. Therefore, cancer treatment remains to be an important target for small molecule and large molecule drug development. The ability to screen compounds or antibodies for their antiangiogenic effects on these signaling pathways, in a rapid and robust way, is critical to current efforts in this area.

Here we describe a method, using HTP® technology to quantify cellular kinase activity without overexpression of kinase or its substrate. Two specific kinases were included in the study.

1. Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), one of multiple subtypes of the receptor kinase Vascular Endothelial Growth Factor, is involved in mediation of almost all cellular responses to VEGF.

2. Signal Transducer and Activator of Transcription 3 (STAT3), acts as a transcription activator, and is phosphorylated by receptor-associated kinases in response to cytokines and growth factors.

Detection of phosphorylated kinase involves a sandwich immunoassay which includes an anti-phospho-specific antibody coupled with an anti-total antibody labeled with either dyes or radioactive labels. The anti-phospho antibody is specific for the phosphorylated epitope in the VEGFR2 in HTF signal, whereas inhibition will demonstrate the opposite effect.

A collection of all assay components were performed in a high throughput format using a non-contact dispenser. Simultaneous, rapid detection of the fluorescent signal from the donor and acceptor fluorophores allows the use of multiple ligands or inhibitors through the combination of a high-performance Xenon flash lamp and dual matched photomultiplier tubes. This technology allows for the quantification of the kinase activity of thousands of cells in parallel, providing a large presence of phosphorylated kinase, upon excitation of the Cy3-streptavidin, energy is transferred to the donor Cy5-streptavidin, allowing for the presence of the phosphorylated kinase, no energy a transfer, and no signal is detected.

HTRF Cellular Kinase Assay

Figure 1 – The HTRF phospho-STAT3 (Tyr705) and phospho-VEGFR2 (Tyr1175) assays are designed for detecting and studying activated kinases directly in whole cells. Using receptor activators, the kinase(s) of interest are activated and the ratio of RBE to ABE monitored. After the lay down of the cells in a 384-well plate, the kinase assay is performed by adding a mix of ATP, an anti-phospho-specific antibody labeled with an RBS-emitting fluorophore, an anti-total antibody labeled with an ABE-emitting fluorophore, and 100% DMSO. The fluorescence ratio is read upon excitation of the RBS-streptavidin, energy is transferred to the donor RBE-streptavidin, allowing for the presence of the phosphorylated kinase, no energy a transfer, and no signal is detected.

Materials and Methods

Materials

A431 cells (Catalog No. 630042) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Dulbecco's Modified Eagle Medium-Ham's F12 Medium (DMEM-F12) (Catalog No. 11040-058) was purchased from Life Technologies (Carlsbad, CA). Antibody (Catalog No. AF357) was purchased from R&D Systems (Minneapolis, MN). 384-Well Endothelial Cell Basal Medium Complete (Catalog No. 31321-012) and EGM SingleQuot® (Catalog No. CC-41730) were donated from Endothelial Cell Basal Medium (Catalog No. CC-31321) and EGM SingleQuot® (Catalog No. CC-41730) were donated from Endothelial Cell Basal Medium (Catalog No. CC-31321), respectively. Recombinant Human Vascular Endothelial Growth Factor (EGF, Catalog No. 2-054) was purchased from R&D Systems (Minneapolis, MN). Recombinant Human FGF-2 (Catalog No. 82500) and Recombinant Human Basic Fibroblast Growth Factor (FGF, Catalog No. 2-031) were purchased from ProSpec-Tanygen Inc. (Haverhill, MA). All other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO).

Methods

Cell Propagation: A431 cells were propagated in complete medium, as described above. The cells were seeded 18 hours prior to running the assay by tip pipetting from the flask, replacing with sterile medium, and regapping with serum-free medium (SBM) (DMEM/F12, 1% HSA) at a density of 120,000 cells/mL. The plates were run at a 1:3 ratio.

Cell Concentration and Viability

Cell concentration analysis was performed using the three known STAT3 pathway inhibitors, Ki 8751 and DMH4, as well as a VEGFR2 neutralizing antibody. 5-point titrations were created of inhibitor tested. Data shown represents average and standard deviation for n=4 replicates.

Assay Validation

2′-Factor agonist, and antagonist titration experiments were performed as described with the automated phospho-VEGFR2 assay procedure. Serial 1:2 dilution scheme, starting at a 1X concentration of 100 µM. EGF (Catalog No. 1500233) was purchased from Baxter (Deerfield, IL). HBSS buffer or medium as previously described to create the final assay concentrations.

The results shown in Figure 3A demonstrate that cell concentrations above 10,000 cells/mL do not promote a signal, therefore the 15,000 cells/mL concentration was chosen to allow lower cell proliferation and unstimulated cells.

The data in Figure 7A demonstrates that allowing the cells to recover from plating and propagating for a period of three days, followed by an overnight stimulation for 24 hours, provides the highest assay window for each of the cell concentrations tested (5,000-10,000 cells/mL). Further optimization verified that higher cell concentrations, in addition to the overnight stimulation period, provide larger differences between stimulated and unstimulated cells. The final conditions chosen included a 3 day plating and overnight (18 hour) serum stimulation of 25,000 cells, followed by a 5 minute HGF stimulation.

BioTek Instrumentation

MultiHT® Microplate Dispenser: The dispenser offers fast, accurate plate dispensing capabilities through its two perpendicular and two syringe pumps, with volumes ranging from 500 µL - 300 µL. 300 µL dispenser cell volume allows for high-sample throughput and accuracy and volume in assays of low as 50 µL.

EL406: Combined Water Dispenser: The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ manifold. It also offers reagent dispensing capabilities through the optional syringe pumps, with volumes ranging from 500 µL - 300 µL. The instrument was used for dispensing cell media, media wash and reagent wash, as well as use for dispensing EGF or VEGF assay samples.

Precisio™ Microplate Pipetting System: The Precisio combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used for dilute and dilute inhibitors, as well as transfer dilute inhibitors to the 96-well plate, and a production of the plates to the 384-well plates.

Synergy™ NEO Multi-Mode Microplate Reader: The reader combines a filter-based and microplate-reader-based detection system in one unit. The Synergy reader uses a filter-based system, high-performance scan, fast lens, as well as dual microplate-reader-based systems to simultaneously detect the 650 nm and 540 nm fluorescent emissions from the assay chemistry.

Conclusions

The HTRF phospho-STAT3 and phospho-VEGFR2 assays afford sensitive, functional cell-based formats for the assessment of kinase signaling pathway modulation.

The optimal HTF/protein kinase assay conditions for each receptor can be easily automated in 384-well or 96-well 384-well format using the aspiration and non-contact dispensing capabilities of the EL406 or MultiHT.

The position-based aspiration and filtered-detection mode of the Synergy™ NEO microplate reader is able to simultaneously quantify the emitted signals from the donor and acceptor fluorophores.

The combination of assay chemistry, as well as liquid handling and detection instrumentation, creates an ideal solution for STAT3 and VEGFR2 pathway activators, in addition to small and large molecule inhibitors.

The authors would like to thank Mark Rothberg and Corning Life Sciences for the generous donation of MLM-63 low volume microplates, as well as Stephanie Nolan and Coen Weisbrieger for their donation of Human Umbilical Vein Endothelial Cells and Medium. Both contributed greatly to the success of the project.

Figure 2 – phospho-STAT3 assay optimization results. A: Cell concentration analysis (5,000-20,000 cells/well tested). B: Signal pathway stimulation (100-minute reaction). C: Serum starvation validation for an unstimulated and unactivated cell line.

Figure 3 – Automated phospho-STAT3 assay workflow. HTF signal detection was carried out using high throughput. The results are shown in Figure 3A demonstrating that cell concentrations above 10,000 cells/mL do not promote a signal, therefore the 15,000 cells/mL concentration was chosen to allow lower cell proliferation and unstimulated cells. The final conditions chosen included a 3 day plating and overnight (18 hour) serum stimulation of 25,000 cells, followed by a 5 minute HGF stimulation.

Figure 7 – phospho-VEGFR2 assay validation results. A: Cell plating and serum starvation time (2 and 3 day plating/hour and overnight). B: Cell concentration and stimulation time (2 and 3 day plating/hour and overnight).

Figure 8 – Assay validation results. A: Data shown represents average and standard deviation of two replicates tested each kinase concentration. B: Assay run on in agonist mode as previously described. Forty-five replicates of 600 nM and 0 nM EGF used as positive and negative control, respectively.

Table 1: STAT3 inhibition (IC50 values)

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Log IC50 vs pIC50 for STAT3 pathway inhibitor IC50 values.

Table 2: VEGFR2 inhibition (IC50 values)

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Log IC50 vs pIC50 for VEGFR2 pathway inhibitor IC50 values.