Using Antibody Arrays for Profiling Angiogenic Factors, RTK Phosphorylation, and Off-Target Responses of Potential Angiogenesis Inhibitors

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THE PROBLEM

It is difficult to quickly screen the effects of potential antiangiogenic agents on multiple secreted angiogenic factors and phosphorylated receptor tyrosine kinases (RTKs) when using time-consuming traditional techniques such as Western blot.

THE SOLUTION

Proteome Profiler™ Antibody Arrays can rapidly detect multiple secreted factors and screen pharmacological responses of angiogenesis inhibitors. There are no gels to run and no proteins to transfer. If you can collect data from an immunoblot, you have the equipment necessary to run an array experiment today. For details please visit RnDSystems.com/ProteomeProfiler.
Arrays that can simultaneously detect multiple proteins increase the amount of information collected from a single experiment. They can reveal changes in protein expression or phosphorylation in context, providing a more complete understanding of the overall intracellular response to treatment. Proteome Profiler Antibody Arrays are designed to simultaneously measure the expression of multiple proteins in a single sample. These multianalyte assays are available in either a membrane-based or a microplate-based format. The membrane-based assays are ideal for profiling a wide range of proteins within a small number of samples and require no specialized equipment beyond what is used for data acquisition when employing Western blot. The microplate-based assays require a small sample size and are amenable to high-throughput analysis of a specific set of cellular proteins. Both assay formats can be performed in approximately 3.5 hours.

To highlight the arrays’ utility, we profiled the relative expression of 55 angiogenesis-related proteins in supernatants from several breast cancer cell lines using the Proteome Profiler Human Angiogenesis Antibody Array. We also screened a panel of VEGF R inhibitors to assess their effects on RTK phosphorylation using the Proteome Profiler Human Phospho-RTK Antibody Array and Proteome Profiler 96 Phospho-RTK Antibody Arrays. Our results showed variations in angiogenic factors secreted by the various breast cancer cell lines and revealed that inhibitors varied in their effectiveness of blocking RTK phosphorylation in each cell line.

MATERIALS AND METHODS

Arrays are composed of capture and control antibodies spotted in duplicate on either nitrocellulose membranes or on the bottoms of transparent 96-well polystyrene plates. For both array formats, cell supernatants or extracts were diluted, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with either the Proteome Profiler Human Angiogenesis Antibody Array (Catalog # ARY007), the Proteome Profiler Human Phospho-RTK Antibody Array (Catalog # ARY001B), the Proteome Profiler 96 Human Phospho-RTK Array1 (Catalog # AR2001), or the Proteome Profiler 96 Human Phospho-RTK Array 3 (Catalog # AR2003). Streptavidin-HRP and chemiluminescent detection reagents were applied to the arrays, and the signal produced at each capture spot corresponded to the amount of protein bound.
Additional Kits and Reagents

ELISA kits from R&D Systems were used to confirm protein or phosphorylated RTK levels: Human Phospho-VEGF R2/KDR DuoSet® IC ELISA Development System (Catalog # DYC1766), Human Angiogenin Quantikine® ELISA Kit (Catalog # DAN00), Human VEGF Quantikine ELISA Kit (Catalog # DVE00), and Human TIMP-1 Quantikine ELISA Kit (Catalog # DTM100). Recombinant RTK ligands from R&D Systems used included: Human VEGF_{165} (Catalog # 293-VE), PDGF-BB (Catalog # 220-BB), EGF (Catalog # 236-EG) and NRG1-β1/HRG1-β1 (Catalog # 396-HB). Tocris Bioscience small molecule inhibitors included: Axitinib (Catalog # 4350), DMH4 (Catalog #4471), (E)-FeCP-oxindole (Catalog # 3882), (Z)-FeCP-oxindole (Catalog # 3883), Ki 8751 (Catalog # 2542), and ZM 323881 hydrochloride (Catalog # 2475).

RESULTS

Profiling Secreted Angiogenic Factors in Supernatants from Breast Cancer Cell Lines

Profile analysis of 55 angiogenic factors in the supernatants from the T47D, MCF-7, and MDA-MB-453 human breast cancer cell lines demonstrated that all three cell lines secreted multiple angiogenesis-related proteins (Figure 1A). Comparison of the relative expression levels of VEGF, Angiogenin, and TIMP-1 in the supernatants from the three breast cancer cell lines revealed that the highest levels of VEGF were present in MDA-MB-453 supernatant while the TIMP-1 was highest in T47D and MCF-7 supernatants (Figure 1B). Concentrations of VEGF, Angiogenin, and TIMP-1 in the breast cancer cell supernatants were also determined using Quantikine ELISAs. The semi-quantitative results obtained from the Proteome Profiler membrane array were comparable to protein concentrations obtained from the ELISAs (Figure 1B).

Conditioned Media from Breast Cancer Cells Phosphorylates VEGF R2

Conditioned media from various breast cancer cell lines were tested for their ability to induce RTK phosphorylation. HUVEC human umbilical vascular endothelial cells were treated with conditioned media from MCF-7, T47D, MDA-MB-468, and MDA-MB-453 human breast cancer cell lines, and cell extracts were then analyzed for RTK phosphorylation. Conditioned media from all four breast cancer cell lines induced phosphorylation of VEGF R2; however, the highest levels of phospho-VEGF R2 were found in the extracts of HUVEC cells treated with conditioned media from MDA-MB-453 cells (Figure 2A). VEGF R2 phosphorylation in HUVEC cell extracts was also analyzed using a DuoSet IC ELISA Development System. Results obtained from the two detection methods were comparable (Figure 2B).
**Measuring Efficacy and Off-Target Responses of VEGF R Inhibitors**

HUVECs were used to generate a VEGF-induced phosphorylation profile and to assess the effects of several small molecule VEGF R inhibitors (Figure 3). The results indicate the varied levels of efficacy of the different inhibitors. Axitinib and Ki 8751 were the most potent inhibitors of VEGF R1 and VEGF R2 phosphorylation. However, DMH4, (E)-FeCP-oxindole, and (Z)-FeCP-oxindole displayed little or no effect on VEGF-induced phosphorylation of VEGF R1 and VEGF R2. Axitinib also inhibited VEGF R2 phosphorylation induced by conditioned media from the MDA-MB-453 human breast cancer cell line (Figure 4). The effects of Axitinib appear to be specific to VEGF R1 and VEGF R2. Axitinib did not block the phosphorylation of PDGF-α or PDGF-β induced by PDGF-BB, EGF-induced EGF R phosphorylation, or the phosphorylation of ErbB2, ErbB3, or ErbB4 induced by NRG1-β1/HRG1-β1 (Figure 5).

**Figure 2.** Phosphorylation of VEGF R2 by Media from Cultured Breast Cancer Cells. HUVEC human umbilical vascular endothelial cells were treated for 5 minutes with conditioned media from the MCF-7, T47D, MDA-MB-468, and MDA-MB-453 human breast cancer cell lines. Phosphorylated VEGF R2 in HUVEC cell extracts was detected using the Proteome Profiler Human Phospho-VEGF R2/ KDR DuoSet IC ELISA Development System. Spot images of Proteome Profiler membrane arrays and the corresponding histogram profiles (gray bars) are shown. The optical densities obtained from the DuoSet IC ELISA (blue bars) are also shown. Results obtained using these detection methods are comparable.

**Figure 3.** Induction and Inhibition of RTK Phosphorylation in HUVECs. HUVEC human umbilical vascular endothelial cells were untreated or treated with 100 nM of the VEGF R inhibitors Axitinib, DMH4, (E)-FeCP-oxindole, (Z)-FeCP-oxindole, Ki 8751 or ZM 323881 hydrochloride for 45 hours. Cells were subsequently treated with 50 ng/mL Recombinant Human VEGF165 for 5 minutes or remained untreated. Phosphorylated VEGF R1 and VEGF R2 in cell extracts were detected using the Proteome Profiler Human Phospho-RTK Antibody Array. Images of Proteome Profiler membrane arrays are shown.
CONCLUSIONS

The Proteome Profiler Human Angiogenesis Array and the Proteome Profiler and Proteome Profiler 96 Human Phospho-RTK Arrays are economical alternatives to traditional methods for screening differences in soluble protein expression and phosphorylation. The membrane-based and plate-based array assays are easy to perform. Both can be completed in approximately 3.5 hours, making them far more time-effective than performing multiple IP-Western blots. In addition, these assays employ a chemiluminescence detection method, thus, no specialized equipment beyond what is typically used to collect Western blot data is required. These arrays are also sensitive enough to compare changes in the phosphorylation of RTKs caused by both ligand and inhibitor treatment, making them extremely useful for performing screens to assess the efficacy and/or off-target effects of potential pharmacological agents.

REFERENCES