Profiling RTK Phosphorylation using Membrane and Plate-Based Arrays

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BACKGROUND

Receptor tyrosine kinase (RTK) expression and phosphorylation is frequently associated with the formation and metastatic spread of cancerous tumors. Considerable effort has been dedicated to the development of inhibitors to target specific RTKs and disrupt aberrant signaling pathways associated with disease states. The goal of this study was to measure the effects of a panel of small molecule inhibitors on RTK phosphorylation using both membrane and plate-based phospho-RTK antibody arrays as screening tools.

METHODS

In this study, RTK antibody arrays were used to simultaneously monitor increases or decreases in the phosphorylation of numerous RTKs in a single sample. Capture antibodies were carefully selected for each kinase and printed on either nitrocellulose membranes approximately the size of a microscope slide or on the bottoms of transparent 96-well polystyrene plates. In both array formats, phosphorylated and unphosphorylated RTKs present in a lysate sample are captured by discrete antibodies. After washing, the arrays are incubated with anti-phosphotyrosine-HRP, which sandwiches with phosphorylated RTKs captured on the array. Following a second wash step, the arrays are incubated with chemiluminescent reagents. Signal generated at each array spot is proportional to the amount of phospho-protein bound by each capture antibody. Recombinant proteins were from R&D Systems: Human FGF Acidic (Catalog # 232-FA), Human NRG-β1/HRG-β1 (Catalog # 396-HB). Inhibitors were from Tocris, an R&D Systems Company: PD 161570 (Catalog #3724), PD 166285 (Catalog # 3785), Gefitinib (Catalog # 3000), PD 173074 (Catalog #3044), HDS029 (Catalog # 2646), PD 153035 (Catalog # 1037), and PD 158780 (Catalog # 2615).

RESULTS

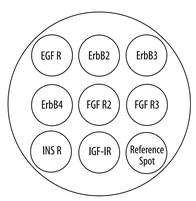
The effects of RTK inhibitors on the phosphorylation of 49 different RTKs was measured using MDA-MB-453 breast cancer and KATO III gastric carcinoma cells. The activation of ErbB2, ErbB3, and ErbB4 in MDA-MB-453 cells was selectively inhibited by HDS 029, PD 153035, or PD 158780. In Kato III cells, the phosphorylation of FGF R2 and EGF R was inhibited by PD 173074, PD 161570, and PD 166285. Although these inhibitors are known to affect the phosphorylation of FGF R family members, these results demonstrate the utility of the Human Phospho-RTK array for monitoring the effect that a specific concentration of inhibitor may have on other RTKs, such as EGF R in this case. Eight different RTKs were identified from the membrane array screening experiments and printed in plate based arrays to assess the dose response of inhibitors.

CONCLUSIONS

The Human Phospho-RTK Array is an economical alternative to traditional methods such as Western blot for screening changes in RTK phosphorylation. Both array assays required 2.5 hours of hands-on time, making this method far more time-effective than performing multiple IP-Western blots. By employing a chemiluminescence detection method, no specialized equipment beyond what is typically used to collect Western blot data was required. Both arrays are sufficiently sensitive to compare changes in phosphorylation caused by both ligand and inhibitor treatment. This method also allows the evaluation of inhibitor selectivity to off-target RTKs.

CONTENT

Well Map
Plate-based Array



Membrane Content Membrane-based Array		
ALK	EphB4	PDGF Rα
Axl	EphB6	PDGF Rβ
Dtk	ErbB2	c-Ret
DDR1	ErbB3	ROR1
DDR2	ErbB4	ROR2
EGF R	FGF R1	RYK
EphA1	FGF R2 α	SCF R
EphA2	FGF R3	Tie-1
EphA3	FGF R4	Tie-2
EphA4	Flt-3	TrkA
EphA5	HGF R	TrkB
EphA6	IGF-I R	TrkC
EphA7	Insulin R	VEGF R1
EphA10	M-CSF R	VEGF R2
EphB1	Mer	VEGF R3
EphB2	MSP R	
EphB3	MuSK	

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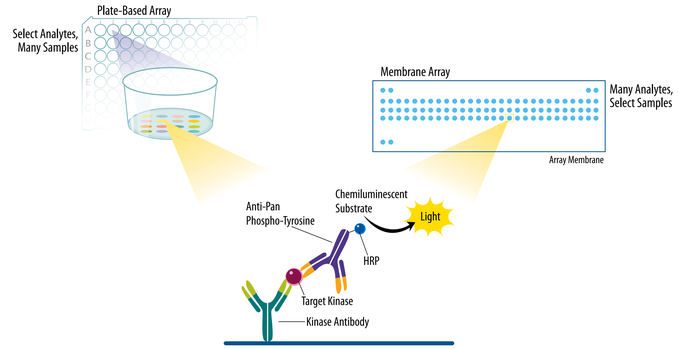

*Suitable imaging systems include Quansys Biosciences Q-View™ Imager; Alpha Innotech Fluorchem® HD2 and FC2; BioRad® VersaDoc™ 4000 or ChemiDoc™ XRS; Fujifilm LAS-3000 or LAS-3000 Mini; Aushon Biosystems SearchLight®, LI-COR Odyssey Infrared Imaging System®, Carestream Image Station 4000MM Pro®. Free analytical software is available.

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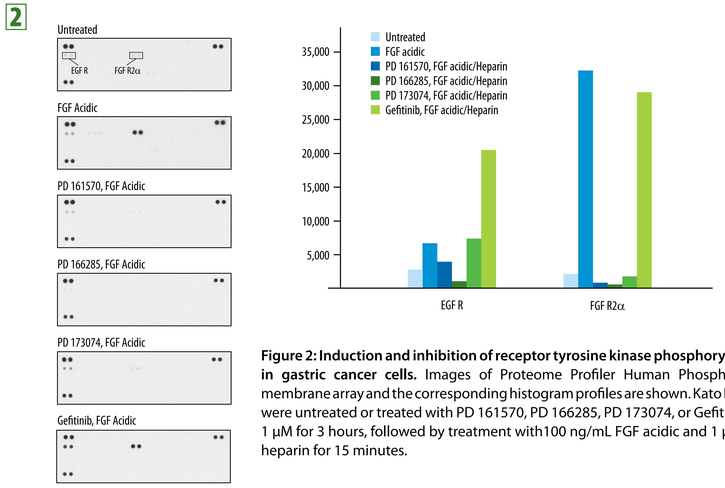

Figure 2: Induction and inhibition of receptor tyrosine kinase phosphorylation in gastric cancer cells. Images of Proteome Profiler Human Phospho-RTK membrane array and the corresponding histogram profiles are shown. Kato III cells were untreated or treated with PD 161570, PD 166285, PD 173074, or Gefitinib at 1 µM for 3 hours, followed by treatment with100 ng/mL FGF acidic and 1 µg/mL heparin for 15 minutes.

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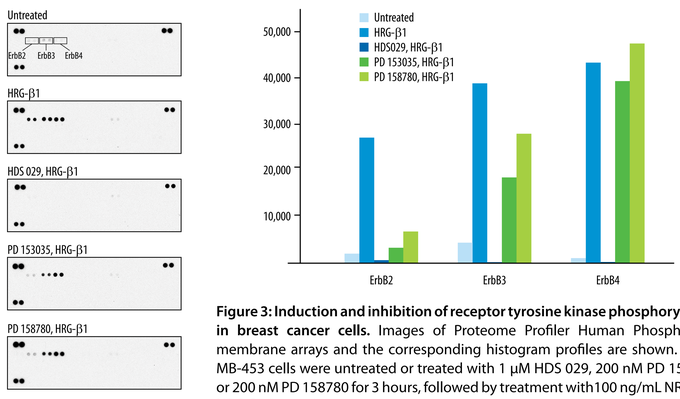
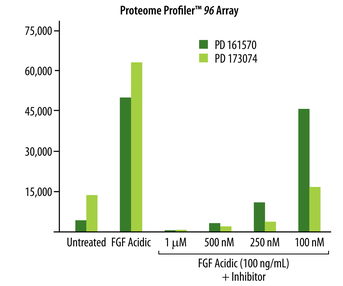


Figure 3: Induction and inhibition of receptor tyrosine kinase phosphorylation in breast cancer cells. Images of Proteome Profiler Human Phospho-RTK membrane arrays and the corresponding histogram profiles are shown. MDA-MB-453 cells were untreated or treated with 1 µM HDS 029, 200 nM PD 153035, or 200 nM PD 158780 for 3 hours, followed by treatment with100 ng/mL NRG-β1/HRG-β1 for 5 minutes.

4A



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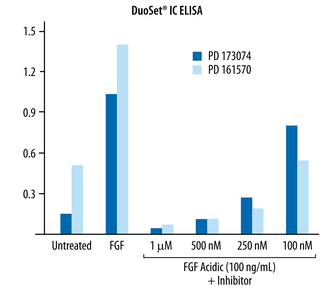

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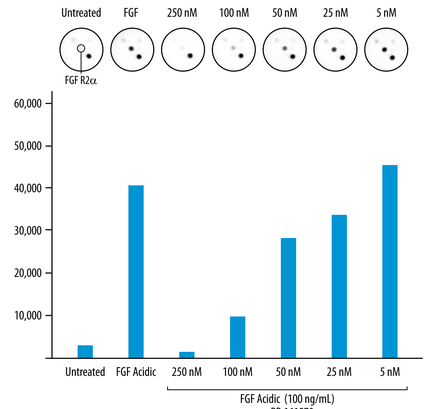

Figure 4: Comparison of phospho-FGF R2 detected using Proteome Profiler *96* Custom Arrays and the DuoSet® IC ELISA. Kato III cells were untreated or treated with PD 161570 over a range of concentrations for 3 hours, followed by treatment with 100 ng/mL FGF acidic and 1 µg/mL heparin for 15 minutes. Histogram profiles of pixel densities from Proteome Profiler *96* Arrays (A) and optical densities measured by DuoSet IC ELISA (B) show both detection methods are comparable. (C) Proteome Profiler *96* Arrays were used to further elucidate the dose response with a narrower concentration range selected from the initial screening results. Well images of each treatment condition are shown along with corresponding histograms.

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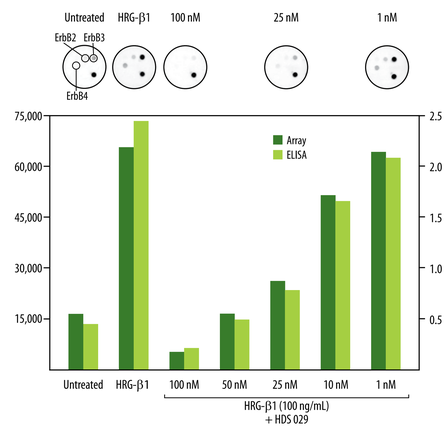

Figure 5: Comparison of phospho-ErbB3 detected using Proteome Profiler *96* Custom Arrays and the DuoSet® IC ELISA. The MDA-MB-453 cell line was treated with PD 161570 over a range of concentrations to determine the dose response of ErbB3 phosphorylation. Cells were treated with inhibitors for 3 hours, followed by treatment with 100 ng/mL NRG-β1/HRG-β1 for 5 minutes. Pixel densities obtained from the Proteome Profiler *96* Arrays (dark green bars) and optical densities obtained from the DuoSet IC ELISA (light green bars) are shown. Results obtained using these detection methods are comparable.

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