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Assay for Isolation of Inhibitors of Her2-Kinase Expression

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Summary

Her2 (ErbB2) protein is overexpressed in breast and other solid tumors, and its expression is associated with progressive disease. Current therapies directed toward Her2 either block dimerization of the receptor or inhibit tyrosine kinase activity to disrupt intracellular signaling. However, little is known about alternative mechanisms for suppressing Her2 expression, possibly by inducing degradation or blocking synthesis. Here, we describe a hybrid western-blotting and enzyme-linked immunosorbent assay (ELISA) designed to identify in low- to medium-throughput format noncytotoxic compounds that reduce expression of Her2 protein.

Keywords

Breast cancer; Heat-shock protein 90 (Hsp90); Her2 (ErbB2) tyrosine kinase; Whole-cell immunoblot

1. Introduction

Human cancers frequently express high levels of transmembrane tyrosine kinases of the Her family, comprising four closely related transmembrane receptor tyrosine kinases, Her1-4 (1-3). Dysregulation of the activity of these receptors often leads to cellular transformation and cancer. These receptors act as a layered network, in which the four receptors are activated by ten growth-factor ligands of the EGF/neuregulin family, to bring about formation of homo- and heterodimers with different signaling capabilities. The first level of regulation of the network relates to formation of the primary signaling unit, which depends on the proper temporal and spatial coexpression of a ligand and two receptors. The major signaling pathways activated by this signaling unit of the Her-receptors include the Ras-Raf1-Mek-Erk, and the PI3K-PDK1-Akt pathways, both of which culminate in activation of transcription programs, as well as cyclin-dependent kinases, leading to progression through the cell cycle (Fig. 1). While Her1 (EGFR), Her3, and Her4 each bind multiple ligands, Her2 (ErbB2, Neu) is a ligandless receptor that enhances and prolongs signaling upon heterodimerization with other Her-receptors. Its overexpression has been detected in up to 30% of breast and ovarian cancers, but also in other types of cancers including lung, gastric, and oral cancers, and has been correlated with invasive and poor prognostic features (1-3). In addition, Her2 has been implicated in mediating increased resistance to chemotherapeutic agents. Current drugs targeted at Her2 receptors work either by blocking receptor dimerization (antireceptor antibodies such as Herceptin) or by inhibiting tyrosine kinase activity (TK inhibitors) (4,5). Identification of novel means to regulate the expression of this kinase should provide additional clinical opportunities for successfully interdicting signaling through Her2-containing receptor complexes.

The stability of Her2 is regulated by the chaperone Hsp90, suggesting inhibition of Hsp90 as an alternative way to block the activity of the kinase (6). Hsp90 activity can be inhibited with agents that bind to its N-terminal region ATP/ADP pocket, such as geldanamycin (GM) and radicicol (RD). Addition of these agents to cells induces the proteasomal degradation of Her2 (7). HDAC inhibitors that acetylate Hsp90 also cause Her2 degradation (8). Thus, agents that target Hsp90 through diverse mechanisms can alter Her2 expression.

An assay designed to identify or evaluate compounds that reduce Her2 levels is represented later (*see* Note 1). This is a low- to medium-throughput *cytoblot-type* assay based on a nonhomogeneous ELISA-type readout that detects endogenous cellular Her2 levels (9) and has been validated by us in the evaluation of Hsp90 inhibitors (approximately 300 compounds) (10,11). In principle, the assay may test compounds that affect kinase transcription, translation, or stability.

2. Materials

2.1. Cell culture and Compound Dispersion

1. The human cancer cell line SKBr3 is maintained in 1:1 mixture of DME:F12 supplemented with 2 mM glutamine, 50 units/mL penicillin, 50 units/mL streptomycin, and 5% heat inactivated fetal bovine serum (FBS) (Gemini Bioproducts, West Sacramento, CA), and incubated at 37°C in 5% CO₂. Stock cultures are grown in T-175 flasks containing 30 mL of DME (HG, F-12, nonessential amino acids, penicillin, and streptomycin), glutamine, and 10% FBS.
2. Cells are dissociated with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) without calcium and magnesium to provide experimental cultures.
3. Using a multichannel pipettor, SKBr3 breast cancer cells are plated at 3,000 cells/well in 100 µL of growth medium in the 96-well microtiter plates, and allowed to attach for 24 h at 37°C and 5% CO₂ (*see* Note 2). Some wells are left without cells to serve as the blank control.
4. Clear-bottom 96-well microtiter plates (Corning Incorporated, Lowell, MA).
5. Multichannel pipettors.
6. SKBr-3 cells (American Type Culture Collection, Manassas, VA) (*see* Note 3).
7. Dulbecco's modified Eagle's medium (DMEM) with Ham's nutrient mixture F12 (DMEM/F12) supplemented with 30 mM d-glucose and nonessential amino acids (Gibco/Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (FBS, Gemini Bio-Products) and Pen-Strep solution (10,000 U/mL penicillin G and 10 mg/mL streptomycin; Gemini Bio-Products).
8. Solution of trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA; 0.02%) in PBS (Gibco/Invitrogen).

¹The assay uses cells, media, buffers, and antibodies that are commercially available, and bulk amounts of these reagents can be purchased at reduced costs. Used at a 1:200 dilution, approximately 45-µL antibody is used for one 96-well plate at 100-µL assay solution/well. At retail price, one vial of antibody (\$128 for 1 mL) would be sufficient to test over twenty 96-well plates, bringing the cost to roughly \$5.50/plate.

²These conditions were optimized for the Analyst plate-reader and the stock of SKBr3 available in our lab. However, considering the variability among cell stocks and the different sensitivity of luminometers, it is highly recommended to perform cell-number optimizations as described in (9). A 48-h attachment period may result in much better retention of cells during washes, thus lower well-to-well variability. If removing the liquid from the microtiter well plates manually using an eight-channel aspirator, position the pipet tips to the leftmost corner of each well to avoid accidental removal of attached cells. Cells are attached quite firmly, but pipetting should be done gently. This is usually achieved by letting liquid fall down the sides of wells, rather than directly into them.

³SKBr3 breast cancer cells overexpress Her2, facilitating its detection in the cytoblot format.

9. Compound library plated in microtiter plates and dissolved at a known concentration in DMSO (Sigma-Aldrich, St. Louis, MO) (*see* Note 4).
10. Positive control: an Hsp90 inhibitor such as radicicol (Sigma-Aldrich) or geldanamycin (Sigma-Aldrich) (*see* Note 5).

2.2. Assay Development and Detection

1. Vacuum source attached to an 8-channel aspirator used to remove liquid from the microplates.
2. Pipet basin (ThermoFisher, Waltham, MA).
3. Ice-cold Tris-buffered saline (TBS) containing 25 mM Tris-HCL, 0.13 M NaCl, 0.0027 M KCl (Fisher Scientific, Fair Lawn, NJ) supplemented with 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA) (*see* Note 6).
4. Methanol, chilled to -20°C (Fisher Scientific) (*see* Note 7).
5. Antibodies: anti-Her2 (c-erbB-2) antibody (Zymed Laboratories/Invitrogen), goat anti-rabbit HRP-linked antibody (Santa Cruz Biotech, Santa Cruz, CA), and normal rabbit IgG (Santa Cruz) (*see* Note 8).
6. ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).
7. Blocking solution, SuperBlock (Pierce Biotechnology, Rockford, IL) (*see* Note 9).
8. Bicinchoninic acid reagent (BCA; Pierce).
9. Standard solution of bovine serum albumin (BSA; Pierce).
10. Rocking platform.
11. Analyst GT plate reader (Molecular Devices, Sunnyvale, CA) (*see* Note 10).

⁴DMSO levels in the final assay volume should not exceed 0.7% (v/v) because the organic solvent is toxic to SKBr3 cells.

⁵The stability of Her2 is dependent on the Hsp90 protein chaperone system, and Hsp90 inhibitors induce the degradation of Her2 (6,7). IC₅₀ values for the commercially available Hsp90 inhibitors GM and RD were determined in the Her2-cytoblot method to be 17 and 28 nM, respectively, consistent with values determined by Western blot analyses (9).

⁶TBST is stored at RT but placed on ice at least 30 min prior to use to allow for chilling.

⁷Methanol should be cooled to -20°C before use.

⁸Antibodies, according to manufacturers' instructions, are stored at 4°C.

⁹Both the ECL Western Blotting Detection Reagents (1 and 2) and the Superblock are stored at 4°C.

¹⁰As of 2007, www.globalspec.com lists 46 companies that make microplate readers. The choice for selecting a certain instrument may be determined by the assay type to be performed, the desired detection sensitivity, and by the available funds. Most screening facilities have several multimode plate readers that can handle 96-well, 384-well, or 1536-well assay plates and support multiplate operations, either through stackers or robotic integration. Perkin Elmer (www.perkinelmer.com) offers several multilabel integratable plate readers for HTS. Among these are VICTOR3™, EnVision™, and ViewLux™. VICTOR3 is a multilabel, upgradable plate reader for fluorescence (top and bottom), luminescence, absorbance, UV absorbance, time-resolved fluorimetry, and fluorescence polarization detection technologies. A small benchtop unit, VICTOR3 operates as a stand-alone instrument or integrated into a robotic system. EnVision™ is a fast, sensitive, multitechnology plate reader designed for fast-paced HTS labs running many different types of assays. The EnVision employs application-specific optical modules, which enables easy customization to meet each user's specifications and needs. The ViewLux is an ultrahigh-throughput microplate imager for lead identification using any of the major assay technologies - FP, FI, TRF, luminescence, radiometric, and absorbance. Molecular Devices (www.moleculardevices.com) also offers several sensitive and versatile multimode readers. Among these are Analyst GT and Analyst HT instruments, which offer detection based on FI, FP, and time-resolved fluorescence resonance energy transfer (TR-FRET), including HTRF, luminescence, and absorbance (epi- and trans-). Each assay detection mode has been optimized to deliver enhanced performance in terms of sensitivity, dynamic range, signal-to-noise, and cross-talk. Analyst instruments support 96-, 384-, and 1536-well plate formats and are optimized for a good integration into robotic environments. Tecan (www.tecan.com) offers the Infinite™ F500 multidetection microplate reader. The Infinite™ F500 is a sensitive filter-based detection platform capable of reading 6- to 1,536-well microplates, including standard- and low-volume 384-well plates. This system supports a wide variety of measurement modes, including FI, absorbance, luminescence (flash and glow), FP, FRET and bioluminescence resonance energy transfer (BRET™), TRF, and TR-FRET-based assays. BioTek Instruments (www.biotek.com) has developed the Synergy™ HT Multi-Detection microplate reader that is compatible with most automated systems. This microplate reader for fluorescence, absorbance, and luminescence measurements was designed with an emphasis on superior performance in all detection methods. The Synergy HT has a 4-Zone™ temperature control system that ensures temperature uniformity necessary for kinetic assays.

3. Methods

The identification of compounds that alter protein levels has required cumbersome in-vitro analyses. This procedure involves treating cultured cells with individual drugs, followed by detergent lysis, polyacrylamide gel electrophoresis of total cellular proteins, and Western blotting to determine protein levels. This methodology is decidedly unsuitable for rapid compound analysis. In contrast, the Her2-blot, which relies on whole-cell immunodetection of the desired proteins, utilizes a minimal number of cells, yet it is sufficiently sensitive and reproducible to permit quantitative determinations. The assay is a hybrid of a Western blot and an enzyme-linked immunosorbent assay (ELISA), and is a modified version of the cyto blot assay developed by Stockwell et al. (12). The cyto blot assay is adaptable for screening numerous protein modifications because the only requirement is a specific primary antibody directed against the protein of interest. It has been applied to HTS to identify many bioactive small molecules, such as inhibitors of mitotic spindle bipolarity (13), mitosis modulators (14), inhibitors of phospho-STAT3 (15), inhibitors of HDAC6-mediated tubulin deacetylation (16), and modulators of the human chromatid decatenation checkpoint (17). Our contribution to the cyto blot is that for the first time we detected a specific decrease in the amount of a protein (9). Testing for the presence of a gene product is far simpler than testing for the absence or near absence of a gene product, because the amount of protein presumably increases with time in the first instance, and thus distinguishes itself from the background. In contrast, when the disappearance of a product is measured, the best resolution from background is at time zero. Notwithstanding this theoretical difficulty, meaningful measurements of Her2 can be obtained.

Figure 2 shows a schematic representation of the assay. The method consists of plating cells in microtiter dishes and treating wells with small molecules at equal concentrations, to identify agents that alter cellular levels of the protein, or at a concentration range to determine their potency in degrading Her2. Following treatment, cells are fixed and permeabilized with methanol. An antibody against the protein and a secondary antibody linked to horseradish peroxidase (HRP) are added. Upon addition of a luminescent substrate, the signal emitted is read in a luminometer. In an assay such as this, where a reduction in signal represents a *hit*, there are potential sources for false positives, such as from cytotoxic agents, which induce a decrease in the signal by cell death rather than through Her2 modulation. Thus, during screening it is important to identify cytotoxic compounds in the library that may reduce total cell number, rather than having a specific effect on Her2. For this reason, the assay can be multiplexed with an indicator of cell viability. A low-cost alternative to viability assays is to determine the amount of total protein in each well using the bicinchoninic acid (BCA) assay.

The Her2-assay described later is designed to include the following controls: vehicle-treated wells (control), wells containing cells treated with geldanamycin or radicicol (positive control; the signal should reach background levels), and wells containing only detection reagent (set as blank in SoftMaxPro software; the values will be subtracted from all analyzed wells). To control for nonspecific antibody binding to the plate, a normal rabbit IgG is added to the plate (background).

1. Dispense the compound library or vehicle (DMSO) to wells, and incubate plates for 12 h (or 6 h) at 37°C and 5% CO₂ (*see* Note 11). Use two wells for the positive control. For geldanamycin and radicicol, the final concentration of the compound in the well is 50 nM.

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¹¹It was determined that addition of an Hsp90 inhibitor to SKBr3 cancer cells induces the rapid proteasomal degradation of Her2, with most protein being depleted at 6 h. To observe maximal effects, a 12-h drug treatment is considered more appropriate. The time frame, however, can be set to an interval that best suits the purpose of the screening assay. However, one should bear in mind that significantly longer treatment periods may increase the potential for cytotoxicity as an artifact in this assay.

2. Remove media through aspiration and wash each well with ice-cold TBST ($2 \times 200 \mu\text{L}$). Use a vacuum source (such as house vacuum) attached to an eight-channel aspirator to remove the liquid from plates.
3. Add cold methanol ($100 \mu\text{L}$ at -20°C) to each well, and incubate the plate at 4°C for 10 min.
4. Wash each well with TBST ($2 \times 200 \mu\text{L}$) to remove the methanol, and incubate at RT for 2 h with 200- μL Super-Block.
5. Add the anti-Her-2 (c-erbB-2) antibody ($100 \mu\text{L}$; 1:200 in SuperBlock) to each assay well and a normal rabbit IgG ($100 \mu\text{L}$; 1:200 in SuperBlock) to nonspecific-binding control wells and incubate the plate overnight at 4°C with gentle rocking.
6. Wash each well with TBST ($2 \times 200 \mu\text{L}$), add the anti-rabbit HRP-linked antibody ($100 \mu\text{L}$; 1:2,000 in SuperBlock), and incubate the plate at RT for 2 h.
7. Wash with TBST ($3 \times 200 \mu\text{L}$) to remove excess antibody (*see* Note 12).
8. Add ECL^{TM} Western Blotting Detection Reagents 1 and 2 in a 1:1 mixture ($100 \mu\text{L}$) (*see* Note 13). Read the plate immediately in an Analyst GT plate reader. Scan each well for 0.1 s. Luminescence height = 0.6 mm; maximum integration = 100,000 μs .
9. Settings: readings per well = 1; target CV per well = 1.0%; raw data units = counts/sec; attenuation mode = out; motion settling time = 15 ms; luminescence aperture = 384/96 aperture; max cps = 100,046.9922 cps; min counts = 349 counts.
10. Import and analyze data in SoftMaxPro (Molecular Devices). Subtract readings from IgG control wells from all measured values. Calculate % Her2 level as luminescence readings resulting from drug-treated cells/untreated cells (vehicle treated) $\times 100$ (9). Fig. 3 shows representative raw data obtained from a Her2-blot (*see* Note 14).
11. To determine the protein content in each well, wash plates from Her2-blot readings with TBST ($2 \times 200 \mu\text{L}$) and incubate them with the BCA reagent ($150 \mu\text{L}$) for 30 min at 37°C . Add a standard solution of BSA to the blank wells.
12. Read absorbance using the Analyst GT (Molecular Devices). Instrument settings: read sequence = row; mode sequence = well; detection mode = a9; excitation side = top; emission side = top; lamp = continuous; top dichroic = 50:50 beam splitter; readings per well = 1; integration time = 100,000 μs ; motion settling time = 200 ms; Z height = 5.33 mm, middle; excitation filter = fluorescence 530 nm. Import and analyze data in SoftMaxPro (Molecular Devices) and determine the protein content of each well (*see* Note 15).

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¹²It is critical to make sure that residual secondary antibody is completely washed out before adding ECL reagents. Any nonspecific residue will create outliers among the data.

¹³For optimal reading results, when adding the ECL Western Blotting Reagents, it is recommended to remove significantly sized air bubbles. These can be gently popped using pipet tips or needles. This is a tedious process, and it is best to take great care to avoid the introduction of bubbles to begin with. Depending on the detection equipment, bubbles may drastically reduce signal intensity.

¹⁴All *hit* compounds must be rescreened. Dose-dependent measurements should be conducted and IC₅₀ values calculated for each *hit* to validate and rank the *hits* generated in the primary screen.

¹⁵Alternatively, putative hits may be retested for cytotoxicity using the sulforhodamine B (SRB) colorimetric assay as described in the protocol (www.sigmaaldrich.com/sigma/bulletin/tox6bul.pdf). The assay is used for cell-density determination, based on the measurement of cellular protein content. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods; however, the method requires only simple equipment and inexpensive reagents.

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Fig. 10.1.

Ligand binding to inactive monomeric Her receptors is followed by dimer formation and kinase activation. Upon recruitment into heterodimers, the ligandless coreceptor Her2 enhances and prolongs signaling through several pathways, including the indicated mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) route, leading to activation of the Akt/PKB kinase. Points of intervention by current drugs targeting Her2 receptors (Herceptin, tyrosine kinase inhibitors) are indicated. The abbreviations used are as follows: *PDK-1* phosphoinositide-dependent kinase 1, *TK* tyrosine kinase.

Fig. 10.2.
Schematic of a Her2-blot.

Fig. 10.3. Representative results of a Her2 blot. Readings obtained in wells containing SKBr3 cells treated for 12 h with DMSO only (control) (*average* 141715.3; *s.d.* 8573.8), 50 nM radicicol (RD) (*average* 9815.3; *s.d.* 2459.1), and 50 nM geldanamycin (GM) (*average* 6467.4; *s.d.* 1256.7), respectively.