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Microtiter Cell-Based Assay for Detection of Agents that Alter Cellular Levels of Her2 and EGFR

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Summary

Overexpression of the transmembrane tyrosine kinases Her2 and EGFR is associated with aggressive malignancies, and several therapeutic strategies targeting the two receptors are now in various stages of clinical development. Most of the known agents block the activation or inhibit the activity of the kinases; however, a more significant therapeutic outcome may result from degrading these oncoproteins. Here, we report the development of a microtiter cell-based assay that sensitively detects cellular levels of Her2 and EGFR. The assay is useful in identifying small molecules that alter cellular levels of these kinases and in quantifying their effect. The method gives results comparable to Western blot, but it is faster, less labor intensive, and amenable to high throughput.

Introduction

Human carcinomas frequently express high levels of transmembrane tyrosine kinases in the human epidermal growth factor receptor (Her) family, and overexpression of at least two of these, Her1 (EGFR) and the closely related Her2, is associated with a more aggressive clinical behavior [1–3]. Transfection or activation of high levels of these two receptors in nonmalignant cell lines can lead to a transformed phenotype [4, 5]. The importance of the Her-receptor family in tumor pathogenesis and progression suggests that agents that interfere with their function may have significant therapeutic activity [6]. Monoclonal antibodies (MAbs) that block activation of the EGFR and Her2 have been developed. Trastuzumab (Herceptin), a humanized anti-Her2 MAb, has clinical activity and was recently approved for use in patients with advanced breast cancer with high levels of Her2. IMC-C225, a chimeric anti-EGFR MAb, has shown activity in combination with chemotherapy or radiation. In addition to antibodies, compounds that directly inhibit the receptor tyrosine kinase activity of Her2 and EGFR have shown preclinical and clinical activity [7].

Recently, it has been shown that some of the irreversible Her2 tyrosine kinase inhibitors enhance ubiquitylation and accelerate endocytosis with subsequent intracellular destruction of Her2 molecules. The degradative pathway stimulated by these agents is mediated by chaperones and is common to the hsp90-interfering compounds [8]. In animals, the irreversible kinase inhibitors were found to have increased antitumor effects, suggesting that destruction, not solely inactivation, of Her2 might have an enhanced therapeutic effect [9].

An alternative way to block the activity of the Her kinases involves targeting hsp90 with agents that interfere with its chaperone function. Hsp90 activity can be inhibited with agents that bind to its N-terminal ATP/ADP pocket, such as geldanamycin (GM), radicicol (RD), and the novel inhibitors PU3 and PU24FCl [10–14] or agents that bind to a C-terminal pocket, such as novobiocin [15]. Addition of these agents to cells induces the proteasomal degradation of a small subset of proteins involved in signal transduction, such as steroid receptors, Raf1 kinase, and certain transmembrane tyrosine kinases such as Her2 and EGFR [10–15]. FK228, a depsipeptide that acetylates hsp90, also causes Her2 degradation [16]. Thus, agents that target hsp90 through diverse mechanisms can cause destruction of Her2 and EGFR.

Identification of new agents that induce the degradation of Her2 and EGFR could lead to novel therapeutic strategies. However, to our knowledge there are no reported assays that can detect cellular levels of Her2 and EGFR in high-throughput format.

A miniaturized whole-cell immunodetection assay was reported by Schreiber et al. [17]. They demonstrated the use of this assay to monitor biosynthetic processes, such as DNA synthesis, and posttranslational processes, such as acetylation and phosphorylation. The assay sensitively detected changes in specific cellular macromolecules in mammalian cells and is compatible with screening large numbers of small molecules in nanoliter to microliter culture volumes.

Here, we modify and extend the use of this technique to the development of a fast, cell-based assay that enables the quantitative analysis of intracellular levels of Her2 and EGFR. We refer to these assays as Her2 blot and EGFR blot, respectively, and apply them to identify and quantify the potency of small molecules in altering cellular levels of these kinases.

Results and Discussion

To identify agents that alter the cellular levels of two of the Her-family members, Her2 and EGFR, we developed a microtiter cell-based assay that is a hybrid Western blotting and enzyme-linked immunosorbent assay (ELISA). The method consists of plating cells in microtiters and treating them with small molecules at equal concentration to identify agents that alter cellular levels of the kinases or varying concentrations of the same compound to determine the potency of the agent in degrading the protein. Following treatment, cells are fixed and permeabilized with MeOH. An antibody against Her2 or EGFR is added, followed by a secondary antibody linked to horseradish peroxidase (HRP). Upon addition of a luminescence substrate, the signal emitted

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is read in a luminometer. The signal of treated and untreated cells is quantified, allowing calculation of IC_{50} values (concentration of drug where 50% of total Her2/EGFR was degraded).

Assay Development for Use in 96-Well Plates

Antibody Optimization

The success of such an assay requires identification of optimal combinations of cell number, primary and secondary antibodies, blocking and washing buffers. We screened a panel of primary and secondary antibodies searching for a reasonable signal to noise ratio and found the combination of two antibodies obtained form Santa Cruz Biotechnology, the anti-Her2 SC-284, a rabbit polyclonal antibody that binds to the carboxyl terminus region of the protein, and the anti-rabbit SC-2004 to perform excellently in the Her2-blot assay (entry 3, Figure 1A). The signal resulting from specific antibody binding (white bars, Figure1A) was significantly greater than nonspecific antibody binding to the plate (black bars, Figure 1A) or cells (gray bars, Figure 1A). For EGFR, the best signal was obtained with the rabbit polyclonal antibody 06-847 from Upstate Biotechnology (entries 1 and 2, Figure 1B). Interestingly, the best performing antibodies in the Her2 and EGFR blot also work well in Western blot assays.

Cell Number Optimization

Experiments were performed with SKBr3, a breast cancer cell line that expresses high levels of Her2, and A431, a vulvar cancer line with EGFR overexpression. As expected, the signal in untreated cells increased with increasing cell number; however, the ratio of signal over background (S/B) reached a plateau at approximately 8000 plated cells/well (Figure 2A). A significant S/B value was obtained using a minimum 1000 cells/well (S/B = 20); however, the calculated Z′ value [18] at this cell number was of a borderline value (Z′ = 0.46). Cell plating numbers above 1000 cells/well were considered useful in a screening assay, the recorded Z′ values and S/B ratios being higher than 0.5 or at least 25, respectively. We believed satisfactory plating to be a number of approximately 3000 cells/well for SKBr3 and approximately 2000 cells/well for A431. A time interval of 48 hr between plating and assay was used to attain a good attachment and confluency of the cells in the well.

Microplate Optimization

Adequate S/B ratios (>30) were obtained using Corning black, clear bottom plates and Perkin Elmer white, clear bottom plates. Paradoxically, the Corning white, clear bottom plates gave higher background readings, and although the signal was additionally high, the maximum S/B value was only 14. Thus, preference was given to Corning black, clear bottom plates, and all data presented here were recorded in such plates.

Assay Test

Z′ Values

A good signal window is essential for hit identification in a HTS assay. Variations associated with vehicle-only treated cells and background IgG measurements are taken in account by the Z′ coefficient. The Z′ parameter is a characteristic of the assay itself, without intervention

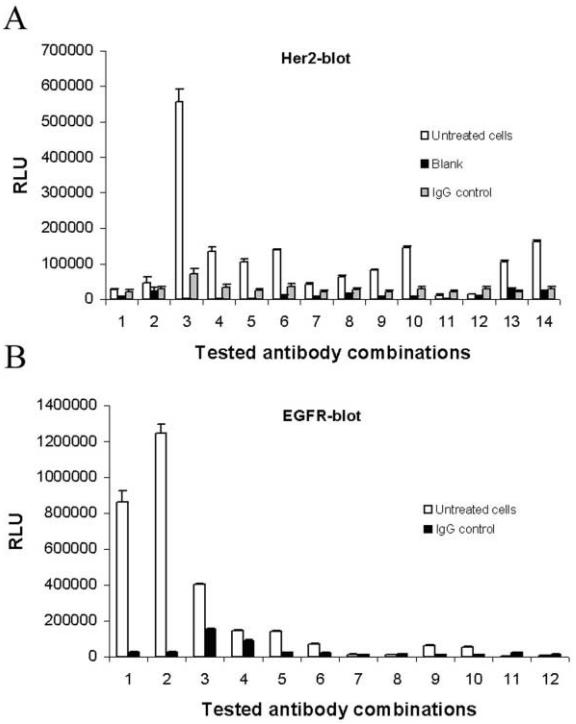

Figure 1. Antibodies Screened for Best Signal/Noise Ratio

(A) SKBr3 cells and (B) A431 cells were treated for 24 hr with vehicle. Cells were fixed and permeabilized, and the Her2 blot (A) or EGFR blot (B) reagents were added. Each column represents the luminescence reading obtained from the combination of a primary and a secondary antibody. Columns: 1, Ab1 + Sec1; 2, Ab2 + Sec2; 3, Ab2 + Sec1; 4, Ab2 + Sec2; etc. For Her2: Ab1 = Transduction Laboratories E19420, mouse IgG2b, epitope = N-terminal domain; Ab2 = Santa Cruz SC-284, rabbit polyclonal, epitope = carboxyl terminus; Ab3 = Oncogene OP39, mouse IgG1, epitope = extracellular domain; Ab4 = NeoMarkers MS-267, mouse monoclonal, epitope = extracellular domain; Ab 5 = NeoMarkers MS-301, mouse monoclonal, epitope = extracellular domain; Ab6 = Oncogene OP16, mouse IgG2a, epitope = extracellular domain; Ab7 = NeoMarkers MS-599, epitope = C terminus. For EGFR: Ab1 = Upstate Biotechnology 06-847, rabbit polyclonal IgG, epitope = C-terminal domain; Ab2 = Upstate Biotechnology 06-129, sheep polyclonal IgG, epitope = cytoplasmic domain; Ab3 = Upstate Biotechnology 05-104, mouse monoclonal IgG, epitope = Ala351-Asp364; Ab4 = BD Pharmingen, mouse IgG; Ab5 = NeoMarkers MS-269-P1, mouse monoclonal, epitope = extracellular domain; Ab6 = Calbiochem PC19, rabbit polyclonal IgG. When Ab is rabbit, Sec1 = Santa Cruz SC-2004 and Sec2 = Sigma A0545. When Ab is mouse, Sec1 = Amersham NXA931 and Sec2 = Sigma A9044. When Ab is sheep, Sec1 = Sigma A3415 and Sec2 = Upstate Biotechnology 12-342. Blank is a measure of unspecific binding of the primary and secondary antibodies to the plate. IgG control represents the reading obtained by addition to cells of a normal IgG and the corresponding HRP-linked secondary antibody.

of compounds. The variability in the obtained signal of the Her2 blot is due to the fact that all reagents were manually added and would likely be diminished with automatization. Even with the introduction of human error, a Z′ value of 0.7 is obtained, a value considered excellent for a screening assay (Figure 2B).

Reproducibility of Data

To test the practicability of our conditions and the accuracy of the Her2/EGFR blot, we tested several agents

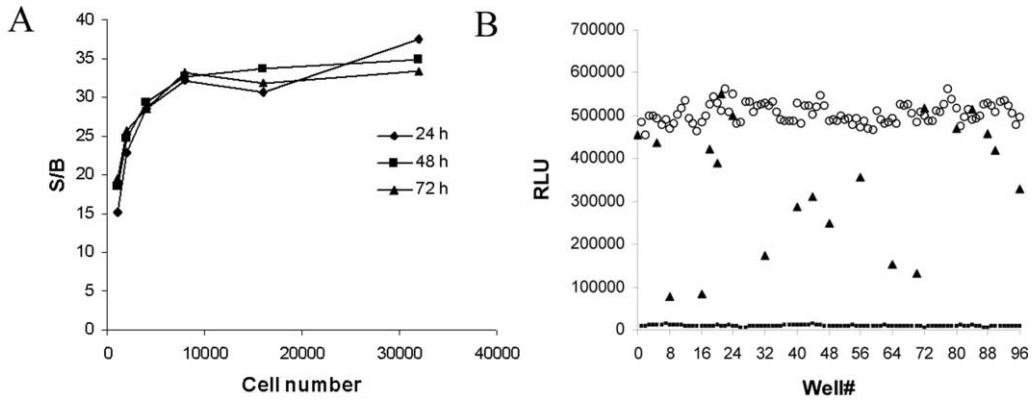

Figure 2. Assay Optimization and Performance

(A) SKBr3 cells were left to attach for the indicated time period prior to Her2-blot analysis. The signal recorded in anti-Her2 (S) and IgG (B) reacted wells was recorded, and the ratio S/B was plotted as a function of cell number. Each measurement represents the average of eight wells.

(B) Results of Z′ analysis in the Her2 blot. SKBr3 cells were reacted with the anti-Her2 antibody (open circles; std = 7.5%), the corresponding IgG (solid squares; std = 16.8%), and 20 hsp90 inhibitors (at 30 μM) from an in-house library (solid triangles) [13, 14].

whose action on Her2 and EGFR was either reported in literature or analyzed in our group by immunoblotting (Western blot). It was determined that addition of an hsp90 inhibitor to SKBr3 cancer cells induced the rapid proteasomal degradation of Her2, most protein being depleted at 6 hr [11] (data not shown). Degradation of EGFR occurs with different kinetics; considerable effects on EGFR in the A431 cell line are observed only at 12 hr [19] (data not shown). To observe maximal protein depletion, a 24 hr drug treatment was considered more appropriate. The time frame, however, can be set to an interval that best suits the purpose of the screening assay.

IC_{50} values for Her2 degradation obtained by our method were compared to Western blot analysis results. The hsp90 inhibitor, PU24FCl, and the natural products GM and RD gave values in the Her2-blot method identical to the Western blot assay. Addition of PU24FCl to SKBr3 cells induces the degradation of Her2 with an IC_{50} value of ~4 μM (Figure 3A). The values obtained with GM and RD were 17 nM and 28 nM, respectively (data not shown), consistent with prior literature reports [11, 12]. The assay was also performed after a 6 hr drug treatment to again reproduce data obtained by Western blot (data not shown).

To rule out that the declining signal in drug-treated cells was not the result of reduced cell number caused by unspecific cell death but to decreased Her2 content, we determined the amount of total protein by the bicinchoninic assay (BCA) [20] and also tested changes in β-actin protein levels. We found the BCA assay to be compatible with the Her2-blot reagents. Two 96-well plates were treated with various concentrations of PU24FCl: the first was subjected to a BCA assay alone, and the second was subjected to a Her2 blot experiment followed by a BCA assay (Figure 4A). Measured differ-

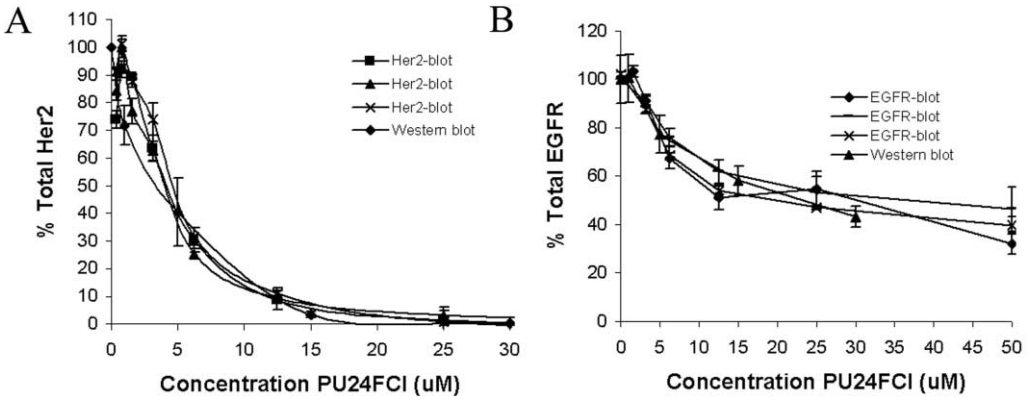

Figure 3. Reproducibility and Robustness of the Her2 and EGFR Blots

(A) SKBr3 cells and (B) A431 cells were treated with various concentrations of PU24FCl for 24 hr in 10 cm plates for Western blot and 96-well plates for Her2/EGFR blot. Her2 or EGFR content was quantified by each method and plotted versus drug concentration. Values obtained from three Her2 blot and EGFR blot experiments conducted on various plates and time intervals are plotted ($p < 0.1$). All values were normalized for total protein content. The Western blot data represent the average of three experiments. In the Her2 blot/EGFR blot assays, drugs were added to quadruplicate wells.

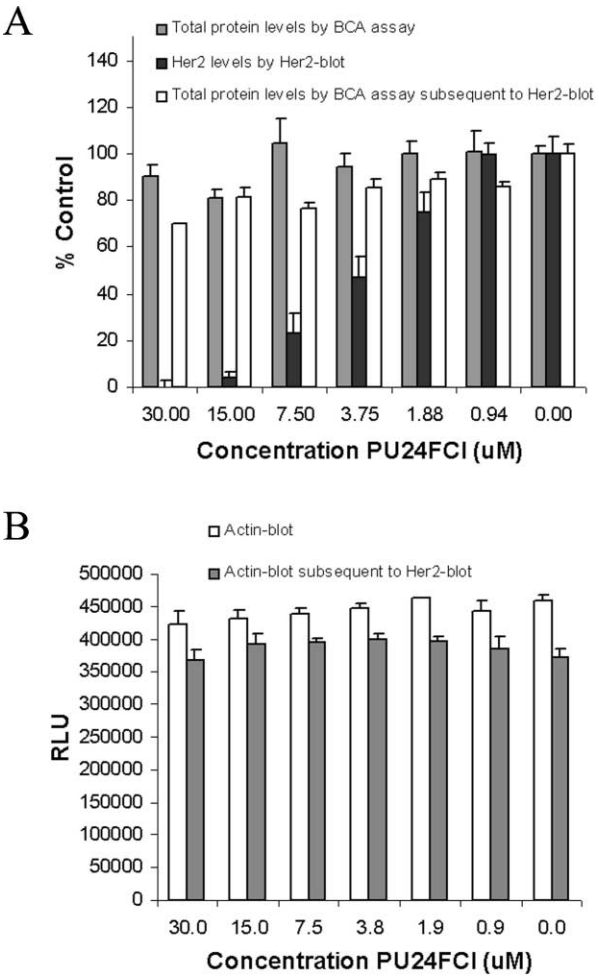

Figure 4. Specificity of the Her2 Blot

SKBr3 cells were treated for 24 hr in two 96-well plates with either vehicle or increasing concentrations of PU24FCl. Cells were fixed and permeabilized.

(A) Total protein concentration was determined using the BCA assay without prior manipulations in the first plate (gray bars) and subsequently to the Her2 blot in the second plate (white bars). Black bars depict Her2 protein levels determined by the Her2 blot. Each measurement is an average of six wells. Control, vehicle-only treated cells.

(B) Changes in β-actin levels were determined upon addition of drug without prior manipulations in the first plate (white bars) and subsequently to the Her2 blot in the second plate (gray bars).

ences in total protein between the two plates were insignificant, and an average of 1.5–1.6 μg protein was detected in each well. We additionally monitored possible changes in β-actin levels due to drug addition, prior to and following the Her2 blot (Figure 4B). If the β-actin blot was performed subsequent to the Her2 blot, plates were stripped and blocked for nonspecific binding prior to the addition of an anti-actin antibody. As expected, the levels of actin in the stripped plate were slightly diminished but detectable. Thus, this method, similar to Western blot, allows for reblotting of the plate. No significant differences in actin expression were seen between untreated and Her2-depleted cells, validating the observation that the lower Her2 levels in treated cells

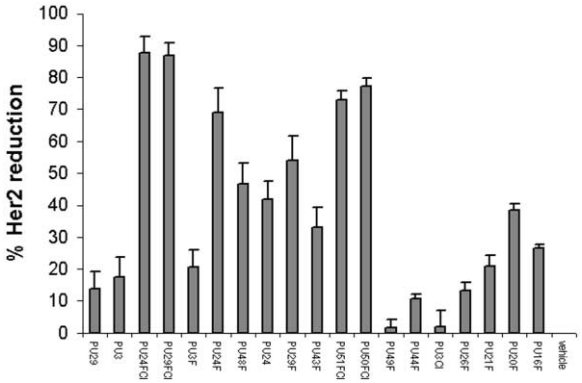

Figure 5. Testing of the Her2 Blot in a Library Screening Format

SKBr3 cells were treated with 20 hsp90-interacting agents [13, 14] (at 30 μM) for 6 hr. The Her2-level-reducing effect of the agents was compared to vehicle-only-treated cells (DMSO). Each measurement is an average of eight wells. The average signal obtained for these agents (in RLU) is plotted in Figure 2B (solid triangles).

were indeed due to a selective reduction in oncoprotein levels. If, however, variation in total protein content is observed, values obtained for Her2 can be normalized to total protein concentration. Both the BCA assay and β-actin blot are compatible with the Her2 blot and can be performed following Her2 quantification. It is noteworthy that actin-level measurements could be indicative of the antiproliferative effect of the tested compounds. Thus, in addition to identifying changes in Her2, screening would be indicative of cytotoxic effects of the compounds.

Stability of the Assay

The Her2-blot assay signal is stable and the obtained values are reproducible from plate-to-plate and day-to-day measurements. PU24FCl was used as a test compound on several plates at different time periods (Figure 3A). The curves obtained in separate readings of this compound were overlapping (IC_{50}s of 4.00, 4.18, and 4.54; standard deviation of 6%).

PU24FCl was also tested in the A431 cell line to determine the feasibility of the assay in quantifying EGFR depletion. Using Western blot and EGFR blot, an IC_{50} value for EGFR degradation of approximately 25 μM was observed. As with the Her2 blot, the EGFR blot was robust, with data obtained by the method reproducible over several plates (Figure 3B).

Assay Test in Library Screen Format

The data presented validate the capability of the assay to quantify the effects of agents that decrease cellular levels of Her2 or EGFR. We further used the assay in a library screen format to identify agents capable of altering the cellular levels of these kinases. A subset of our hsp90-inhibitor library [14] was added to SKBr3 cells at a set concentration of 30 μM, and the ability of these agents to decrease Her2 levels in cells was determined after a 6 hr treatment (Figure 5 and Figure 2B). Agents that alter cellular levels of kinase by at least 25%–30% can be considered "hits" with a high degree of confidence (Figure 2B). There was no significant change in total protein content as determined by the BCA assay;

thus, the values reflect the effect of these compounds on Her2 expression.

It is noteworthy that identical activity in the series of compounds was previously obtained using the traditional Western blot [14]; however, this work required a three-week period.

The presented technique has advantages over traditional Western blot methods in that it is faster and less labor intensive. On the negative side, the assay cannot differentiate between full-length Her kinase and cleavage fragments that would be easily distinguishable on Western blot, as fragments would run differently.

Significance

Given the important role of the Her receptor family in the pathogenesis and progression of cancer, agents that interfere with their activity could have an important role in the treatment of cancer patients. Monoclonal antibodies that block the activation of the kinases or small molecules that inhibit their function are now in different stages of clinical trial or are already in clinical use. These agents do not induce significant kinase degradation, and it is believed that decreasing cellular levels of these kinases may result in a greater clinical activity. We now report a cell-based microtiter-format method for identifying and quantifying agents that alter cellular levels of Her2 and EGFR. Given that this assay uses cells and a nonhomogeneous ELISA-type readout, it is applicable to high throughput. New agents that induce the degradation of Her2 and EGFR can thus be identified by screening large libraries of compounds using this assay format. Considering the potential clinical importance of these agents, this fast and reliable assay may aid new drug development efforts.

Experimental Procedures

Cell Culture

The human cancer cell lines SKBr3 and A431 were obtained from the American Type Culture Collection (Manassas, VA) and 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) and incubated at 37°C in 5% CO_2. Stock culture was grown in T-175 flasks containing 30 ml DME (HG, F-12, nonessential amino acids, and penicillin and streptomycin) with glutamine and 10% FBS. Cells were dissociated with 0.05% trypsin and 0.02% EDTA in phosphate buffer saline (PBS) without calcium and magnesium.

GM was obtained from the Developmental Therapeutics Program/National Cancer Institute, while RD was purchased from Sigma-Aldrich. The synthesis of PU24FCl and the purine-based hsp90 library was described elsewhere [13, 14]. All drug stocks were made in DMSO.

Her2 Blot

Experimental cultures were plated in black, clear-bottom microtiter plates (Corning 3603) (3000 cells per well for SKBr3 and 2000 cells per well for A431) in growth medium (100 μl) and allowed to attach for at least 48 hr at 37°C and 5% CO_2. Some wells were left without cells to serve as the blank. Growth medium (100 μl) with drug or vehicle (DMSO) was carefully added to the wells, and the microtiter plates were placed at 37°C and 5% CO_2. DMSO levels should not exceed 0.1% (higher levels were found to affect the growth of cancer cells).

Following incubation (6 hr or 24 hr), wells were washed twice with ice-cold Tris buffer saline (TBS) containing 0.1% Tween 20 (TBST) (200 μl). A house vacuum source attached to an eight-channel aspirator was used to remove the liquid from the microplates. Further, methanol (100 μl at −20°C) was added to each well, and the plate was left at 4°C for 10 min. Methanol was removed by washing with TBST (2 × 200 μl). The plate was further incubated at RT for 1 hr with SuperBlock (Pierce 37535) (200 μl) and overnight at 4°C with the anti-Her-2 antibody (Santa Cruz Biotechnology SC-284) (100 μl, 1:200 in SuperBlock). Each well was washed with TBST (2 × 200 μl) and incubated at RT for 2 hr with an anti-rabbit HRP-linked antibody (Sigma A0545) (100 μl, 1:1000 in SuperBlock). Unreacted antibody was removed by washing with TBST (3 × 200 μl), and the chemiluminescent substrate solution (100 μl) (Pierce 38040) was added. The plate was read 5 min later in an Analyst AD plate reader (Molecular Devices). Each well was scanned for 0.1 s. Readings from wells containing only control IgG and the corresponding HRP-linked secondary antibody were set as background and deducted from all measured values. Luminescence readings resulted from drug-treated cells versus untreated cells (vehicle treated) were quantified and plotted against drug concentration to give the IC_{50} values (defined as concentration of drug required to degrade 50% of total Her2).

Other anti-Her2 antibodies tested were purchased from Transduction Laboratories (E19420), NeoMarkers (MS-267, MS-301, MS-599), and Oncogene (OP16, OP39). Another anti-rabbit-HRP antibody was purchased from Santa Cruz Biotechnology (SC-2004), and the anti-mouse-HRP antibodies were purchased from Amersham (NXA931) and Sigma (A9044). Normal rabbit and mouse IgGs were purchased from Santa Cruz Biotechnology (SC-2027 and SC-2025).

EGFR Blot

The experiment was conducted identically as for Her2. The anti-EGFR antibody used was purchased from Upstate Biotechnology (06-847) and was used 1:500 in SuperBlock. Other anti-EGFR antibodies tested were purchased from Upstate Biotechnology (06-129, 05-104), Santa Cruz Biotechnology (SC-03-G), NeoMarkers (MS-269-P1), BD Pharmingen (610016), and Calbiochem (PC19). The anti-sheep HRP-linked antibodies and the normal sheep IgG were purchased from Sigma (A3415), Upstate Biotechnology (12-342), and Santa Cruz Biotechnology (SC-2717), respectively.

Actin Blot

If the assay was performed previous to the Her2 or EGFR-blots, the experiment was conducted as described for the Her2 blot. The anti-β-actin antibody was purchased from Sigma (AC15) and was used 1:5000 in SuperBlock. The secondary antibody was purchased from Amersham (NXA931) and used 1:10,000 in SuperBlock. If the assay was performed consequently to the Her2 blot, the plates were washed with TBST (2 × 200 μl) and stripped with Pierce Stripping Buffer (Pierce 21059) (100 μl) for 10 min at room temperature.

Total Protein

Total protein content was determined using the bicinchoninic acid (BCA) reagent (Pierce 23225). Plates resulting from Her2/EGFR blot readings were washed with TBST (2 × 200 μl) and incubated with the BCA reagent (150 μl) for 30 min at 37°C. Protein concentration was determined using a standard solution of bovine serum albumin (BSA). Absorbance was read using a Spectra Max Plus microplate spectrophotometer (Molecular Devices).

Protein Assays

Cells were grown to 60%–70% confluence and exposed to drugs or DMSO vehicle for the indicated time periods. Lysates were prepared using 50 mM Tris (pH 7.4), 0.1% NP-40 lysis buffer. Protein concentration was determined using the BCA kit (Pierce 23225) according to the manufacturer's instructions. Clarified protein lysates (20–50 μg) were electrophoretically resolved on denaturing SDS-PAGE, transferred to nitrocellulose, and probed with anti-Her2 (C-18) (Santa Cruz Biotechnology SC-284) or anti-EGFR (Upstate Biotechnology 06-847) followed by the corresponding HRP-linked secondary antibody. Blots were visualized by autoradiography, and the protein was quantified using BioRad Gel Doc 1000 software. The IC_{50} was

calculated as the drug concentration needed to degrade 50% of the total protein.

Data Analysis

Statistical analysis was performed using the Analysis Tool Pack provided in Microsoft Excel. To detect differences between data sets, a Student's t test (2-tailed) was applied. Values for which $p < 0.15$ were considered significant.

Z′ values were determined as 1 − 3*(StdSample + StdBkg)/(AvgSample − AvgBkg), where StdSample is the standard deviation of the signal obtained from anti-Her2-antibody (or anti-EGFR)-reacted wells, and StdBkg is the standard deviation of the signal obtained from control IgG-reacted cells in the Her2 blot or EGFR blot. AvgSample and AvgBkg are the average of the corresponding signals.

S/B is defined as the ratio of the averaged signals obtained in anti-kinase and control IgG-reacted cells.

Supplemental Data

Raw data (in RLU) for antibody, cell number, and plate optimization tests are available at http://www.chembiol.com/cgi/content/full/10/7/629/DC1.

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