Activity Assay of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Triple-Negative Breast Cancer Cells Using Peptide-Conjugated Magnetic Beads

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ABSTRACT

Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer with limited treatment options. Epidermal growth factor receptor I (EGFR) has emerged as a promising target in TNBC. Limited success of the EGFR kinase inhibiting small molecules in clinical trials may be attributed in part to inaccuracy in identifying EGFR signatures in patient tumors. In light of the absence of a simple correlation between EGFR expression and its degree of activation, a simple and reliable tool that can quantify EGFR kinase activity in tumor samples may be of therapeutic value in predicting patient-specific EGFR targeted therapies. This study reports the development of an assay that can quantitatively profile EGFR kinase activities and inhibitor sensitivities in TNBC cell lysates by using peptide reporters covalently tethered to magnetic beads in a controlled orientation. The use of magnetic beads provides rapid sample handling and easy product isolation. The potential of this approach was demonstrated by screening a set of five clinically relevant EGFR tyrosine kinase inhibitors. Formatted for microwell plates, this magnetic bead-based kinase assay may be used as a complementary approach for direct high-throughput screening of small molecule inhibitors.

INTRODUCTION

Protein tyrosine kinases (PTKs) regulate important cellular processes in normal cells as well as in several diseases, including cancer.[1,2] Realizing the importance of kinases as therapeutic drug targets, several methods have been undertaken to assess protein activity in cancer cells. Conventional assays of kinase activity involved detecting incorporation of terminal phosphate from P^{32}-labeled ATP. Recent years have witnessed the development of several high-throughput kinase assays, including arrays of antibodies, to capture PTKs followed by detection of phosphotyrosine content with secondary antibodies.[3] These methods, however, require antibodies for selective recognition of phosphorylated residues. Arrays of immobilized proteins or peptides have also been developed to profile kinase activity directly. In its simplest form, a kinase assay monitors the phosphorylation of a substrate in the presence of kinase and ATP. Experimentally derived consensus motifs and random peptide libraries have been screened to identify exogenous substrate sequences with high specificity and rates of reactivity.[4,5] Unlike antibody arrays, these peptide microarrays offer multiplexed detection of kinase activity with spatial addressing, thereby facilitating the quantification of multiple kinase activities using a single anti-phosphotyrosine antibody. Diverse approaches have been developed to immobilize protein or peptide substrates onto solid surfaces, including peptide synthesis *in situ*,[6,7] noncovalent attachment via streptavidin–biotin interaction, covalent attachment via chemical ligation, N-hydroxysuccinimide (NHS)–amine reaction, Diel–Alder reaction and Michael addition,[8–12] attachment of self-assembled monolayers of alkanethiols on gold surfaces,[9] as well as copolymerization into polyacrylamide gels.[13,14] Thus, while numerous specific surface-attachment strategies have been devised, none has yet been widely accepted due to inherent challenges in these approaches, including reduced protein activity due to dehydration or denaturation, reduced surface density due to steric hindrance, and difficulty in adaptation to high-throughput screening.

Triple-negative breast cancer (TNBC), lacking estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), poses a serious clinical challenge because of poor prognosis,[15–17] extreme aggressiveness, and metastasis.[16,17] Limited treatment options exist for TNBC and recurrence is common. Hence, several initiatives have been undertaken to identify clinically

ABBREVIATIONS: BSA, bovine serum albumin; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; EDT, 1,2-ethanedithiol; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor 2; HRP, horseradish peroxidase; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; PTK, protein tyrosine kinase; RT, room temperature; S/B, signal/background; S/N, signal/noise; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TKI, tyrosine kinase inhibitors; TNBC, triple negative breast cancer.

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relevant molecular targets in TNBC. Epidermal growth factor receptor (EGFR), which is expressed in around 60% of TNBCs, has emerged as a promising clinical target.[18–20] Even though the use of EGFR tyrosine kinase inhibitors (*e.g.,* gefitinib and erlotinib) has shown promising results *in vitro* with basal-like breast cancer cell lines,[21,22] clinical trials of these drugs in breast cancer have not been as successful.[23,24] This lack of clinical response can be partially attributed to inefficiency in identifying tumors with increased EGFR activity.[18,23,24] A sensitive and reliable method to detect EGFR abnormalities in breast cancer cells would likely identify a cohort of patients who would benefit from EGFR targeted therapies. The absence of a simple relationship between receptor expression and its degree of activation probably led to the failure of the clinical trials, which explored the response to anti-EGFR drugs based only on protein expression.[18] Under these circumstances, profiling EGFR kinase activity signature in breast cancer samples would not only provide a more accurate report of the disease state, but also may be more successful in screening inhibitors and monitoring treatment progression of individual patients. Hence, there is an urgent need of a simple, robust yet cost-effective platform for high-throughput screening of small molecule drug candidates.

Major challenges in developing cell-based PTK assays involve sensitive and specific detection of the activity of one PTK in the background of multiple tyrosine kinases present in cellular extracts and obtaining accurate measurements from small biopsy samples. Solid-phase kinase assays, where either the kinase or the substrate is tethered to solid surfaces, have several advantages over solution/homogeneous phase kinase assays, including easing purification and facilitating scale-down of the assay. In this study, the peptide reporters were covalently tethered to magnetic beads in a controlled orientation via a COOH-terminal cysteine residue on peptide substrates by Michael addition chemistry. The immobilized substrate was then incubated either with recombinant EGFR or cellular extracts of TNBC cells with or without small molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs). Substrate phosphorylation, measured via chemifluorescence, was sensitive and provided selective quantification of kinase activity in cancer cells. This assay also allowed for direct screening of small molecule EGFR kinase inhibitors.

EXPERIMENTAL

Preparation and Purification of Peptide

Amino acids, CLEAR-Amide resin, and 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium haxafluorophosphate (HCTU) were obtained from Peptides International. Piperidine, N-methylmorpholine, trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), and triisopropylsilane (TIS) were obtained from Sigma-Aldrich. All reagents were used as received without any purification. The peptides KKKAEEEEYFELVAC, SPAFDNLYYWDQDPPERC, KKAFDNPDYWNHSLPPRC, KKKSNFANFSAYPSEEDMC, RRLIEDN EYTARGC, and ADEYLIPQQC were synthesized on an automated synthesizer, Prelude™ (Protein Technologies, Inc.), using a solid-phase method based on Fmoc-chemistry. Cleavage of the crude peptide was performed with the mixture of TFA/ddH_2O/EDT/TIS (94:2.5:2.5:1 v/v) at room temperature (RT). The crude peptide was then precipitated and washed with cold diethyl ether three times. The crude peptides were tested using ABI 4700 MALDI TOF/TOF mass spectrometry (Applied Biosystems) to confirm the correct molecular masses and Agilent 1200 Series LC/MS system for purity. Purification was done through a preparative C_{18} column in the Agilent 1200 LC/MS system if necessary.

Cell Culture and Lysate Production

Human breast cancer (MDA-MB-468 and MDA-MB-231) cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and glutamine, whereas MCF-7 cells were grown in DMEM supplemented with FBS and glutamine (supplemented DMEM). To maintain full length HER2 cDNA transfected MCF-7 (MCF-7/HER2) cells, 10 μg/mL insulin and 0.4 μg/mL G418 were added in supplemented DMEM. During the culture, the media were changed every other day. The cells were passaged every 5–6 days using Trypsin-EDTA (0.25% trypsin, 1 mM EDTA). To lyse the cells, the cells were washed 2× with cold phosphate-buffered saline (PBS), and then 1 mL of cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, and 10% glycerol) containing 1× protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 1 mM activated sodium orthovanadate was added to each cell flask. The cells were incubated on ice for 15 min with occasional swirling, then removed from the plate with a cell scraper, and transferred to a microcentrifuge tube. The cell lysate was then clarified by centrifuging at 14,000 *g* at 4°C for 15 min. Total protein concentration was determined using a BCA protein assay kit (Pierce), and the lysates were stored at −80°C until further use.

Kinase Inhibitors

Gefitinib and erlotinib were procured from LC Labs, CL-387,785 from EMD Chemicals and BIBW-2992 and CI-1033 from Chemietek.

Substrate Immobilization on Bead Surface

COOH terminated magnetic beads were procured from Bioclone. As illustrated in *Figure 1A*, a simple attachment strategy was utilized to covalently immobilize peptide substrates on to the beads. 300 μL of magnetic beads were thoroughly washed. A magnetic stand (DynaMag-2 magnet; Invitrogen) was used to collect the beads. Following incubation with 50 mg/mL of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 50 mg/mL of NHS for 30 min at RT with gentle shaking, the beads were washed 3× with 100 mM MES buffer (pH: 5.0) and resuspended in 10 mM cystamine dihydrochloride solution in the same buffer. The reaction was carried out for 2 h at RT. After the completion of the coupling reaction, the beads were collected, washed, and incubated with 50 mM tris (2-carboxyethyl)phosphine (TCEP) for 30 min at RT. The activated beads were washed again and functionalized with Sartomer SR 415 (Exton) in 100 mM $NaHCO_3$ (pH 8.4) for 30 min. After washing the beads 3× in water, the remaining unreacted thiol groups were blocked with 2 M sodium acrylate in 100 mM Na_2CO_3 for 30 min. Finally, beads were incubated with peptides diluted in a 100 mM

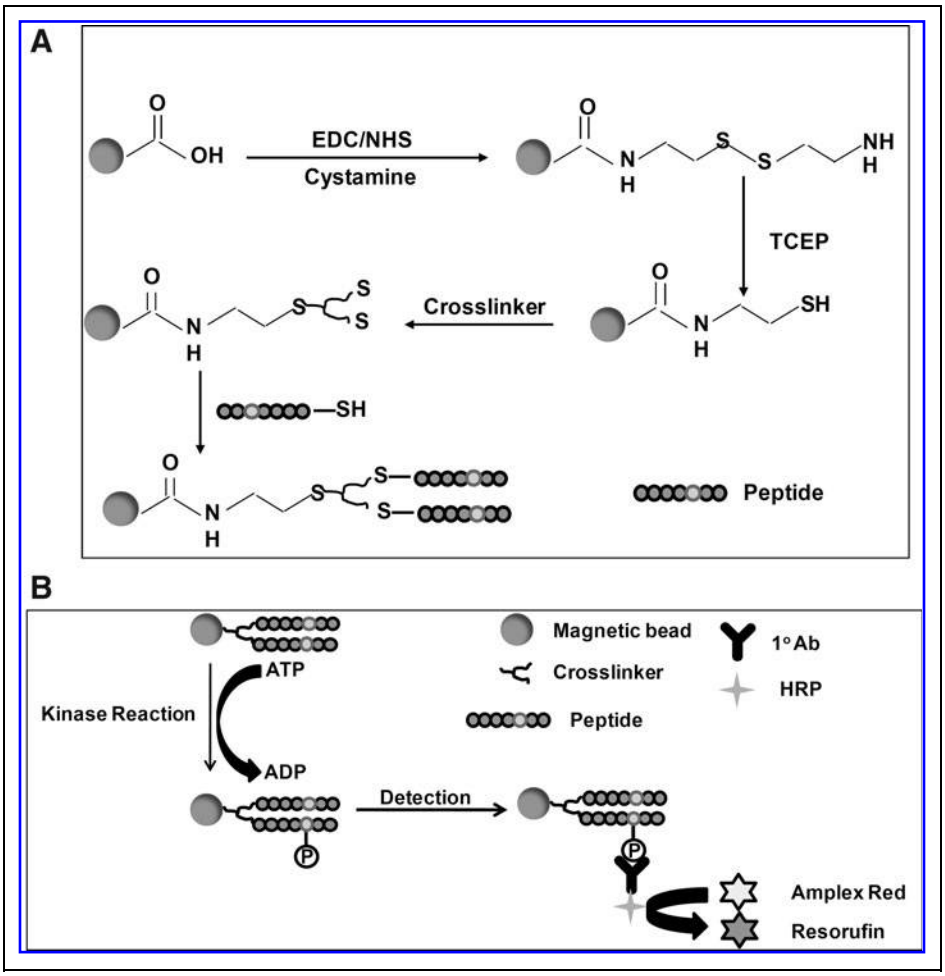

Fig. 1. **(A)** Peptide reporters were covalently attached to the magnetic beads in a controlled orientation. **(B)** Immobilized peptide was then incubated with different reaction mixtures. Quantitative profiling of phosphorylated status of EGFR and sensitivities of EGFR to inhibitors using a magnetic bead assay. Following the reaction, the phosphorylation of the substrates was detected by chemifluorescence. EGFR, epidermal growth factor receptor.

$NaHCO_3$ solution for 3 h at RT. Following the conjugation reaction, the beads were washed in TBST (10 mM Tris-HCl, 100 mM NaCl, and 0.15% Tween-20), and then in water before storing at 4°C.

Evaluation of Loading Capacity of the Beads

100 µL of magnetic beads was conjugated to 100 µL of 550 µmoles/L peptide substrates for 3 h as described earlier. Following the reaction, magnetic beads were separated from the mixture using the magnetic stand and the clear supernatant was collected. The concentration of the unbound substrate in the supernatant was evaluated using the BCA protein assay kit and the difference in concentration of the substrates before and after conjugation reaction was then utilized to calculate the loading capacity of the beads for the substrates.

Array-Based Kinase Assay

To carry out the kinase assay, initially the beads were added to each well of a 96-well plate and blocked with 10% bovine serum albumin (BSA) in the EGFR kinase buffer (20 mM HEPES pH: 7.2, 10 mM $MnCl_2$, 1 mM DTT, 15 mM $MgCl_2$, and 40 µg/mL BSA) for 30 min at RT. The substrate was then incubated with the reaction mixture consisting of recombinant EGFR or cell lysates (at indicated concentrations), 6 µL of 1 mM ATP, 20 µL of 3× EGFR kinase assay buffer, 6 µL of 50% glycerol and water (total volume of 60 µL) at RT. Typically 5 µL of substrate-conjugated beads was used for each reaction. EGFR-TKIs at varying concentrations were incubated with cell lysates for 30 min at RT before performing the kinase assay.

Detection and Quantification of Phosphorylated Substrates

After the reaction, the beads were washed with TBST and blocked in TBST containing 1% BSA for 1 h at RT. This was followed by incubation of the beads with horseradish peroxidase (HRP)-conjugated 4G10 anti-phosphotyrosine (Millipore) antibody for 1 h at RT. The beads were then washed 3× with TBST and resuspended in 50 mM sodium phosphate (pH: 7.5) buffer. For chemifluorescent detection, 50µL of freshly prepared 50µM Amplex red and 1 mM H_2O_2 (in the sodium phosphate buffer) was added to each well. The fluorescent intensity was detected using a Quad-4-monochromator microplate reader (Tecan Safire 2) with an excitation wavelength of 532 nm and an emission wavelength of 590 nm. Dose–response curve fits were generated (3 parameter fit in GraphPad Prism) by plotting fluorescence units/s against log concentration of inhibitors.

Statistical Analysis for Assay Evaluation

To statistically analyze the dynamic range and reproducibility of the assay, a fluorescent signal obtained upon incubation with the MDA-MB-468 cell lysate was considered the positive control, whereas that obtained from the EGFR-immunodepleted MDA-MB-468 supernatant was regarded as the negative control. The signal/background (S/B) ratio was calculated as μ_{pos}/μ_{neg}, whereas the signal/noise (S/N) ratio was evaluated as $(\mu_{pos} - \mu_{neg})/\sigma_{neg}$. μ_{pos} and σ_{pos} are the mean and standard deviation of positive controls, whereas μ_{neg} and σ_{neg} are mean and standard deviation of negative controls, respectively. The *Z*-factor, a dimensionless number which represents both the dynamic range and variability across multiple samples, was calculated from $1 - [3 \times (\sigma_{pos} + \sigma_{neg})/|\mu_{pos} - \mu_{neg}|]$.[25]

The *Z*-factor ranges from negative values to 1, but values greater than 0.5 are considered to be adequate.

Cell Viability Assay

MDA-MB-468 cells were seeded at a density of 5×10^3 cells/well in 96-well plates. After 24 h, erlotinib, gefitinib, CL-387,785, CI-1033, and BIBW-2992 at varying concentrations were added and the cells were incubated further for 48 h. The cells were then washed with PBS and cell viability was measured using a XTT assay kit (Sigma). A dose–response curve fit was generated (3 parameter fit in GraphPad Prism) by plotting inhibition of cell growth against log concentration of inhibitors.

RESULTS AND DISCUSSION

To develop a simple yet robust and reproducible assay for monitoring EGFR activity and inhibitor sensitivities, peptide substrates were covalently immobilized onto the magnetic beads. After kinase reactions, the beads were washed and phosphorylation of the peptides was detected by chemifluorescence using an HRP-conjugated primary antibody against phosphorylated tyrosine (*Fig. 1B*). The fluorescence intensity measured is directly proportional to substrate phosphorylation, which in turn is proportional to EGFR kinase activity in cancer cells.

Development of Kinase Assay with Substrate-Immobilized Magnetic Beads

Six different peptides specific for the EGFR kinase (*Table 1*) were designed based on either the optimal EGFR substrate sequence or sequences that had been previously shown to be efficiently phosphorylated by EGFR *in vitro*.[26–30] To increase substrate accessibility, and hence peptide density on the bead surface, substrates were immobilized on the beads via a cross linker, Sartomer SR415. Earlier studies in our group directed at evaluating the effect of different cross linkers on substrate accessibility showed that SR415 markedly increased peptide density as compared to bisacrylamide and other branched PEG linkers.[11] Cystamine was conjugated to COOH-terminated magnetic beads utilizing EDC/NHS chemistry. Reduction of disulfide bonds by TCEP generated free thiol groups, which were then conjugated to the cross linker SR415. Substrates were covalently attached to the cross linker by Michael addition of the sulfhydryl group at the COOH-terminal cysteine. As described in Materials and Methods, the concentration of unbound peptide was calculated using a BCA protein assay kit, and the difference in peptide concentration before and after conjugation reaction was utilized to estimate the loading capacity of the beads. The loading capacity was estimated to be 15×10^{-17} moles of peptide substrate/bead.

To test the peptide substrate accessibility to EGFR and detection of antibodies as well as the sensitivity of detecting substrate phosphorylation, substrate-immobilized beads were incubated with serially diluted purified recombinant EGFR for 1 h in the presence of ATP and substrate phosphorylation was evaluated via chemifluorescence.

Table 1. Sequences of Different EGFR Kinase-Specific Peptides

Peptide number	Sequence	Reference
1	KKKAEEEEYFELVAC	26–28
2	SPAFDNLYYWDQDPPERC	26,27
3	KKAFDNPDYWNHSLPPRC	26,27
4	KKKSNFANFSAYPSEEDMC	28,29
5	RRLIEDNEYTARGC	26,27
6	ADEYLIPQQC	26,27

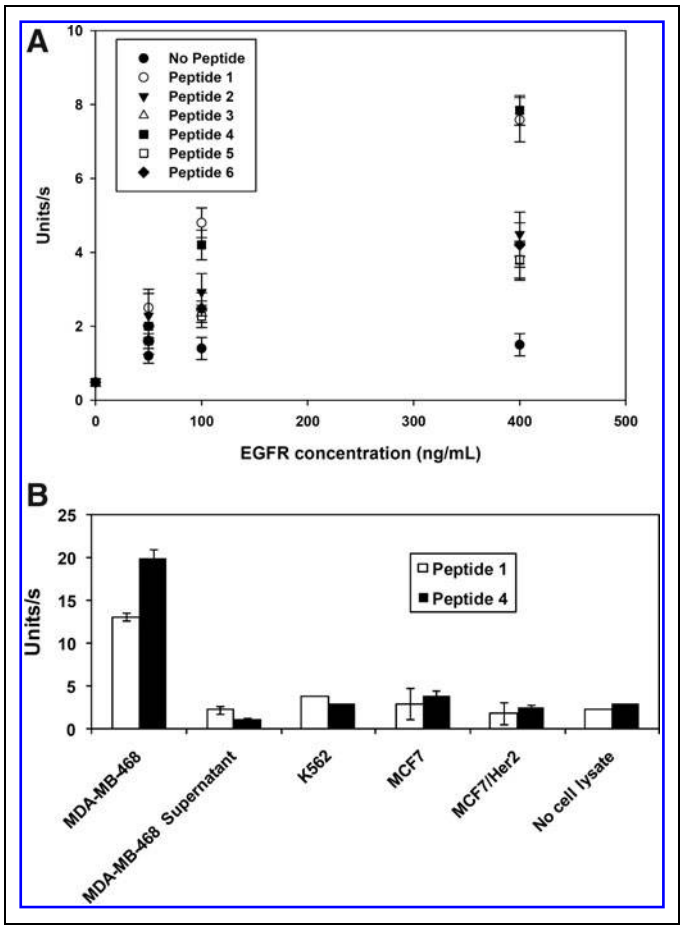

Fig. 2. (A) Selection of peptide substrate. Peptides 1–6 (*Table 1*) were immobilized onto the magnetic beads and incubated for 1 h with 0–400 ng/mL of purified recombinant EGFR. Comparison of substrate phosphorylation following incubation of beads with or without peptide substrates with varying concentration of EGFR. Each data point represents the mean of three independent experiments in which each experiment contained three replicates. The error bars represent SEM ($n = 3$). **(B)** Specificity of phosphorylation of immobilized peptides 1 and 4 by EGFR. Substrate-conjugated beads were incubated for 1 h with 100 μg of MDA-MB-468, EGFR-immunodepleted MDA-MB-468 supernatant, K562 lysate, MCF-7, MCF-7/Her2, and no lysate control and substrate phosphorylation was detected by chemifluorescence. Each data point represents the mean of three independent experiments in which each experiment contained three replicates. The error bars represent SEM ($n = 3$).

As a control, EGFR was added to beads lacking immobilized peptides. The significant difference in fluorescent intensity obtained from beads with and without substrates (*Fig. 2A*) demonstrated the potential of this magnetic bead-based assay to detect peptide phosphorylation with high sensitivity. Of the peptide substrates tested, significantly higher signals were obtained for peptide 1 and peptide 4 than the other peptides ($P < 0.05$ at 100 and 400 ng/mL EGFR), indicating peptides 1 and 4 were more efficiently phosphorylated by EGFR *in vitro*.

Major challenges in developing a kinase assay include identifying a highly specific substrate and sensitively detecting phosphorylation of this substrate because cancer cell lysates contain multiple tyrosine kinases, which can potentially contribute toward the phosphorylation of the substrate. So, to evaluate the specificity and detection sensitivity of EGFR phosphorylation of peptides 1 and 4, the substrates were incubated for 1 h with 100 μg of cellular extracts of the TNBC cell line, MDA-MB-468 (TNBC with high EGFR expression), MCF-7 (luminal breast cancer with a very low EGFR expression),[31,32] MCF-7/HER2 (MCF-7 cells expressing HER2),[33] K562 (human chronic myeloid leukemia cell line lacking EGFR expression),[30,34] supernatant of EGFR-immunodepleted MDA-MB-468, and a no-lysate control. As demonstrated in *Figure 2B*, a high signal was obtained for MDA-MB-468 cells in case of both the peptide reporters (13 and 20 units/s for peptide 1 and 4, respectively), as compared to K562, MCF7, and MCF7/HER2 cells (4, 3, and 2 units/s and 3, 4, and 3 units/s for peptide 1 and 4, respectively). Also, very little phosphorylation of immobilized substrates was observed for the EGFR-immunodepleted MDA-MB-468 supernatant and no lysate control (2 and 2 units/s and 1 and 3 units/s for peptide 1 and 4, respectively), indicating that kinases other than EGFR contributed very little toward the phosphorylation of either peptide reporters under these reaction conditions (*Fig. 2B*). Several parameters were calculated to identify the optimal substrate for further development of the kinase assay. Using the signal obtained upon incubating MDA-MB-468 cell lysates as the positive control and that obtained from the EGFR-immunodepleted MDA-MB-468 supernatant as the negative control, the *Z*-factor was calculated to be 0.7 for peptide 1 and 0.75 for peptide 4. This suggested that the dynamic range as well as variability across multiple samples for both the peptide reporters is comparable. However, since the S/B and S/N ratios for peptide 1 were lower than those of peptide 4 (6 vs. 18.2 S/B and 64 vs. 111.2 S/N), peptide 4 was chosen as the substrate for further development.

Substrate Optimization

Initial studies were directed toward optimizing the kinase reaction conditions. To assess the dynamic range of the bead-based assay, peptide 4-conjugated magnetic beads were incubated with serially diluted MDA-MB-468 cell lysates for 1 h. As illustrated in *Figure 3A*, increasing total lysate protein from 0 to 75 μg enhanced substrate phosphorylation. However, further increases in cell lysate amount did not increase substrate phosphorylation significantly. As compared to the no lysate control, a statistically significant difference in substrate phosphorylation was observed ($P < 0.05$) when the protein

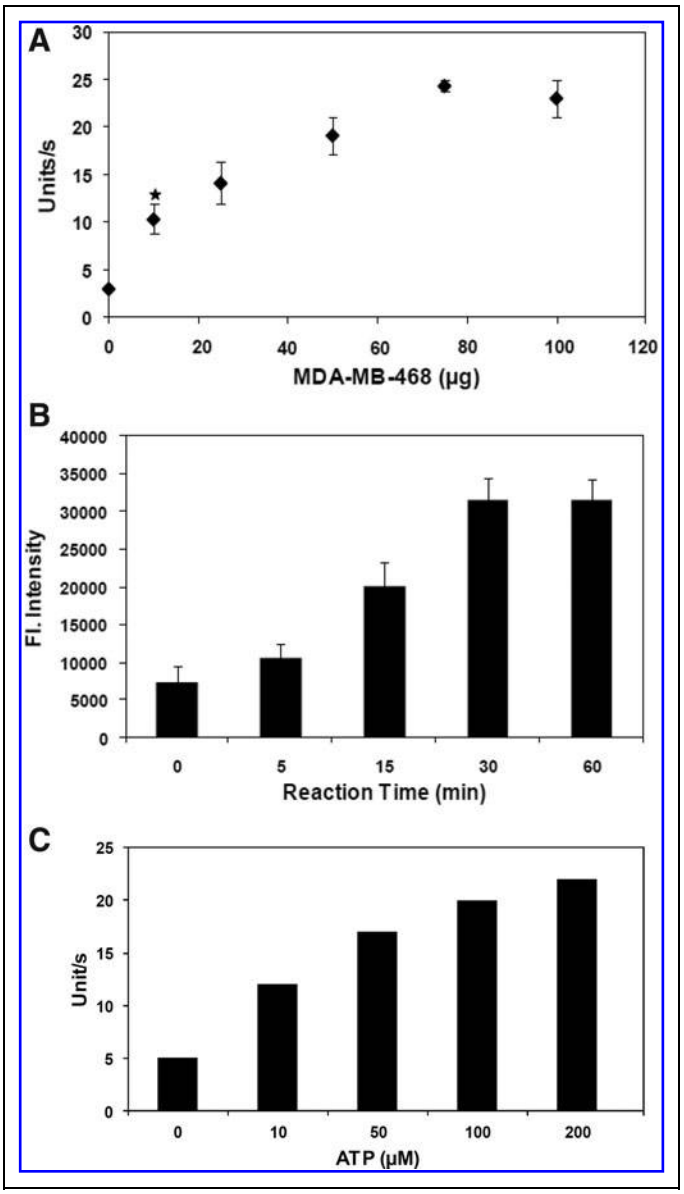

Fig. 3. Optimization of total protein in MDA-MB-468 cell lysate and reaction time. **(A)** 0–100 μg cell lysate was incubated with peptide 4 for 1 h. Extent of substrate phosphorylation was quantified by chemifluorescence and is presented as units/s. Each data point represents the mean of three replicates and the error bars represent SEM. **(B)** Extent of substrate phosphorylation was quantified by incubating peptide 4 with 50 μg of MDA-MB-468 cell lysate. Each data point represents the mean of three independent experiments in which each experiment contained three replicates of each sample, and the error bars represent SEM ($n = 3$). **(C)** Extent of substrate phosphorylation as a function of ATP concentration.

array was incubated with 10 μg cell lysates, which corresponded to approximately 500 cancer cells. Using the signal obtained upon incubation with no lysate as a negative control, *Z*-factors were −0.03, 0.25, 0.7, and 0.8, and S/N ratios were 8.1, 12.1, 27.8, and 33.01, for 10, 25, 50, and 75 μg MDA-MB-468 cell lysate. Since

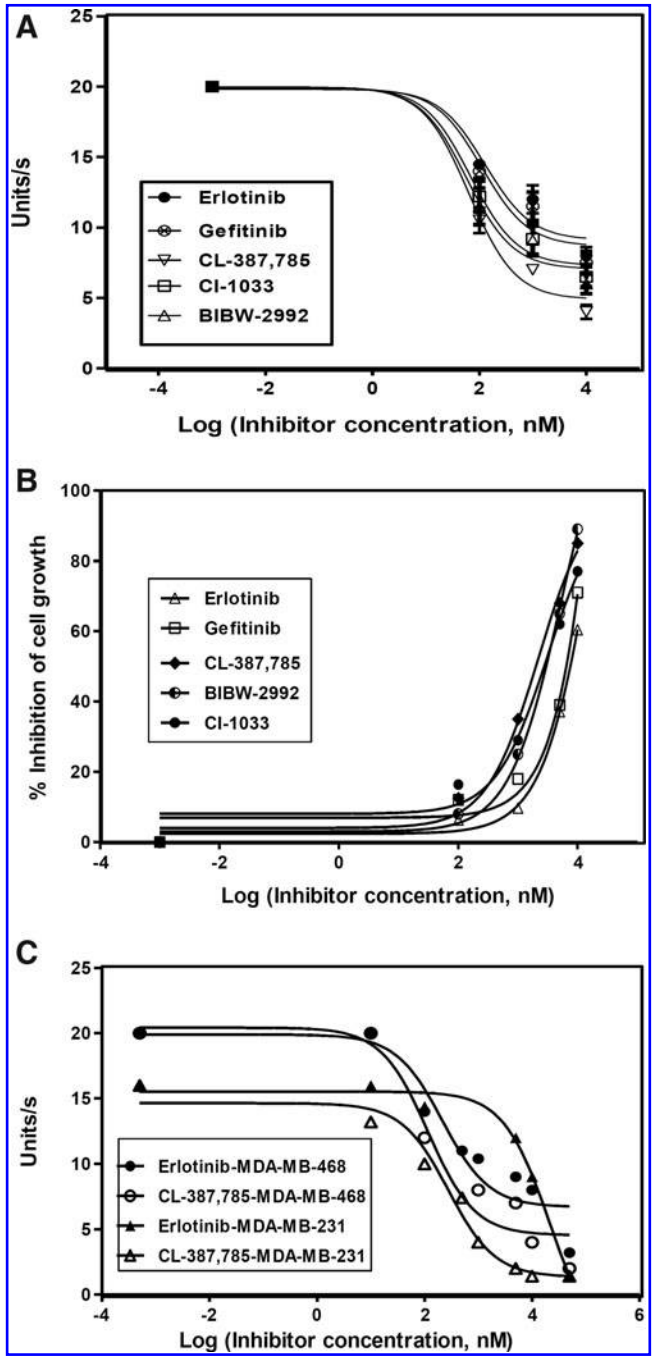

Fig. 4. **(A)** Comparison of the effects different EGFR inhibitors on the phosphorylation of immobilized peptide 4 upon incubation with 50 μg of MDA-MB-468 cell lysates for 30 min. **(B)** Comparison of the effect of different EGFR inhibitors on the viability of MDA-MB-468 cells. **(C)** Comparison of the effect of erlotinib and CL-387,785 on substrate phosphorylation when incubated with MDA-MB-468 and MDA-MB-231 cells. Each data point represents the mean of three independent experiments in which each experiment contained three replicates. The error bars represent SEM ($n=3$).

Z-factor values and S/N ratios obtained with 50 μg cell lysate were higher than those obtained for 10 and 25 μg and were comparable to those obtained with 75 μg of cell lysate, the phosphorylation of the substrate at different time points (*Fig. 3B*) for up to 60 min was then monitored using 50 μg of cell lysate per reaction. As expected, substrate phosphorylation increased with increased reaction time. However, beyond 30 min, phosphorylation signal saturated, highlighting the reduction in reaction rate as the substrate is depleted. *Z*-factors were 0.4, 0.7, and 0.7, and S/N ratios were 32, 52, and 52, for incubation time of 15, 30, and 60 min. Hence, all subsequent experiments were performed with 30 min incubation in the cell lysate. The concentration of ATP was varied from 0–150 μM. In the absence of ATP, little phosphorylation signal was detected. Increasing the ATP concentration from 0 to 50 μM resulted in an increased phosphorylation signal (*Fig. 3C*). Above 50 μM, further increases in the ATP concentration had no effect on the substrate phosphorylation signal. The ATP concentration used for all subsequent experiments was 100 μM.

Inhibitor Screening in Cell Lysates

After confirming the ability of the magnetic bead-based assay to quantitatively profile EGFR activity status in cellular extracts, experiments were designed to investigate its potential utility in screening clinically relevant kinase inhibitors. For this purpose, peptide 4-conjugated magnetic beads were incubated with MDA-MB-468 cell lysates in the presence of five different ATP competitive inhibitors at concentrations varying from 0.1 to 10 μM. The inhibitors tested include the first generation inhibitors erlotinib and gefitinib (EGFR specific), as well as the second generation irreversible inhibitors CL-387,785 (EGFR specific), CI-1033 (pan-EGFR), and BIBW-2992 (EGFR-ErbB2 dual inhibitor). *Figure 4A* demonstrates the dose-dependent reduction of substrate phosphorylation by each inhibitor. First generation inhibitors, erlotinib and gefitinib, had moderate effects on substrate phosphorylation as compared to the second generation inhibitors. *Table 2* compares the IC_{50} of different inhibitors. Higher potency of second generation inhibitors is probably due to the ability of these inhibitors to covalently bind to the cysteine residue in the ATP binding pocket of the EGFR kinase domain via a Michael addition reaction. The covalent attachments ensure a higher occupancy of the ATP binding site and

Table 2. Comparison of IC_{50} Values of Different EGFR Kinase Inhibitors Between Kinase Assay and Cell Proliferation Assay

	Bead-based assay	Cell proliferation assay
Erlotinib	7.2 ± 2.1	8.5 ± 3.1
Gefitinib	8 ± 3.1	8.1 ± 2
CL-387,785	0.7 ± 0.3	2.2 ± 0.6
BIBW-2992	1.0 ± 0.9	2.2 ± 1.0
CI-1033	1.4 ± 0.8	2.5 ± 0.8

Each data point represents the mean ± SD ($n=3$), expressed in μM.

thus enable these TKIs to inhibit the activation of EGFR. Further experiments were performed to examine the effect of these inhibitors on viability of MDA-MB-468 cells. As shown in *Figure 4B*, inhibitors induced a dose-dependent growth inhibition of the cells, with second generation inhibitors being more efficient than first generation inhibitors. As illustrated in *Table 2*, IC_{50} values obtained for the cell viability assay were comparable to that of the bead-based assay, highlighting the ability of the magnetic bead assay to act as a complimentary approach for direct screening of small molecule inhibitors.

As a further demonstration of the assay as a tool for screening inhibitors, substrate-conjugated beads were incubated with cellular extracts of MDA-MB-231 in the presence of erlotinib and CL-387,785. *Figure 4C* compares the effects of these compounds on MDA-MB-468 and MDA-MB-231 cells. Both of these are TNBC cell lines; however, MDA-MB-231 cells are highly invasive, while MDA-MB-468 cells are not.[35] Both the cell lines showed increased sensitivity toward CL-387,785 [IC_{50} values (μM) of 0.7 ± 0.3 and 0.5 ± 0.2 (CL-387,785) vs. 5.6 ± 2.5 and 7.2 ± 1.5 (erlotinib) for MDA-MB-468 and MDA-MB-231, respectively]. Thus, this study illustrates the potential of the bead-based assay to screen for inhibitors irrespective of the aggressive or metastatic characteristic of cancer.

In summary, we report here the development of a sensitive and robust magnetic bead-based assay to achieve sensitive, selective, and quantitative measurement of EGFR kinase activity in TNBC cells using chemifluorescence detection as read out. The study demonstrated the ability of this assay to monitor EGFR activity and screen for small molecule compounds to inhibit EGFR activity in TNBC cell lines and highlighted the translational relevance of the assay. By incubating substrate tethered beads with lysates prepared from patient-derived fine-needle aspirations, this assay can be used to predict the response of an individual patient to kinase inhibitor therapy based on the decline of kinase activity in the presence of tyrosine kinase inhibitors. Thus, this method may be helpful in identifying cohorts of TNBC patients who would benefit from EGFR kinase inhibitor therapy and predicting appropriate treatment. Furthermore, this assay has the potential to be expanded to multiplexed format for simultaneous quantitative analysis of different kinases, which are crucial for cancer progression and to accurately predict individualized kinase inhibitor cocktails and monitor patient response.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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