Molecular Pharmacology Fast Forward. Published on September 12, 2006 as doi:10.1124/mol.106.027656

MOL Manuscript #27656

Pharmacology and signaling properties of EGFR isoforms studied by Bioluminescence Resonance Energy Transfer (BRET)

Hans H. Schiffer, Esther C. Reding, Stephen R. Fuhs, Qing Lu, Fabrice Piu, Steven Wong, Pey-Lih H. Littler, Dave M. Weiner, William Keefe, Phil K. Tan, Norman R. Nash, Anne E. Knapp, Roger Olsson, Mark R. Brann

ACADIA Pharmaceuticals,

San Diego, California, 92121, USA

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: BRET EGFR assay

Address correspondence to:

Hans H. Schiffer

ACADIA Pharmaceuticals

3911 Sorrento Valley Blvd.

San Diego, California, 92121, USA

Tel: 858 320-8625

Fax. 858 558-2872

E-mail: hschiffer@acadia-pharm.com

Text pages: 39

Tables: 1

Figures: 5

References: 36

Abstract: 244

Introduction: 749

Discussion: 1467

Non-standard abbreviations:

BRET, bioluminescence-resonance-energy-transfer; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; NSCLC, non-small-cell lung cancer; GFP, green fluorescence protein; PLCγ1, phospholipase gamma 1; RTK, receptor tyrosine kinase; Luc, luciferase

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We have developed a new assay for measuring epidermal growth factor receptor (EGFR) activation using the bioluminescence-resonance-energy-transfer (BRET) technology, which directly measures the recruitment of signaling proteins to activated EGFR. Our results demonstrate that EGFR BRET assays precisely measure the pharmacology and signaling properties of EGFR expressed in HEK293T cells. EGFR BRET assays are highly sensitive to known EGFR ligands (pEC_{50} EGF = 10.1 +/- 0.09), consistent with previous pharmacological methods for measuring EGFR activation. We applied EGFR BRET assays to study the characteristics of somatic EGFR mutations that were recently identified in lung cancer. In agreement with recent reports, we detected constitutively active mutant EGFR isoforms, which predominantly signal through the PI3K/Akt pathway. The EGFR inhibitors Iressa or Tarceva are several folds more potent in inhibiting constitutive activity of mutant EGFR isoforms compared to wild type EGFR. Notable, our results reveal that most of the mutant EGFR isoforms tested were significantly impaired in their response to EGF. The highest level of constitutive activity and nearly complete loss of EGF responsiveness was detected in isoforms, which carry the activating mutation L858R and the secondary resistance mutation T790M. In summary, our study reveals that somatic mutation in EGFR quantitatively differ in pharmacology and signaling properties, which suggest the possibility of differential clinical responsiveness to treatment with EGFR inhibitors. Furthermore, we demonstrate that the EGFR BRET assays are a useful tool to study the pharmacology of ligand-induced interaction between EGFR and signaling pathway specifying adapter proteins.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Overexpression and activation of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), plays an important role in the etiology of non-small-cell lung cancer (NSCLC) (Pao and Miller, 2005). Consequently, EGFR is recognized as a key target for the development of NSCLC therapies (Hynes and Lane, 2005). Two drug development strategies focusing on EGFR inhibition are currently pursued: (I) the identification of reversible or irreversible small molecule drugs that inhibit the intracellular tyrosine kinase activity of EGFR by competitively binding to the ATP-binding site of the kinase domain, and (II) the identification of humanized monoclonal antibodies (Mab) that interact with extra-cellular EGFR domains interfering with ligand binding (e.g., epidermal growth factor, EGF) or EGFR dimerization (Hynes and Lane, 2005). The reversible small molecule EGFR inhibitors Iressa (gefitinib) (Herbst et al., 2004) and Tarceva (erlotinib) (Minna and Dowell, 2005) and the antibody drug Erbitux (cetuximab, IMC-C225) (Goldberg, 2005) have already been marketed in the US for NSCLC. However, early clinical studies observed only a 10-15% response rate in a US population of unselected NSCLC patients treated with both drugs (Fukuoka et al., 2003; Perez-Soler et al., 2004). A partial explanation for this low response rate was recently obtained by the discovery of somatic EGFR mutations in only a small subset of NSCLC patients. They clustered in exons 18-21 of the EGFR gene, which encode the intracellular EGFR tyrosine kinase domain (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). The presence of these mutations is significantly associated with a clinical response to treatment with Iressa or Tarceva, giving hope that they could be used as biomarkers to predict drug responsiveness (Pao and Miller, 2005). The somatic mutations cluster in the activating loop of the EGFR kinase domain and have been more frequently found in females, Asians, non-smokers and adenocarcinomas (Pao and Miller, 2005).

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

These mutations include point mutations that change single amino acids and small in-frame deletions (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). A particularly high incidence has been observed in non-smokers that have adenocarcinomas with bronchoalveolar (BAC) features (Pao and Miller, 2005). Importantly, cancer cell lines, which endogenously express high levels of the mutated EGFRs, are significantly more sensitive to growth inhibition when treated with Iressa or Tarceva (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a; Sordella et al., 2004). Several *in vitro* studies show that the somatic mutations identified in EGFR are activating mutations, causing aberrant activation of downstream EGFR signaling pathways and an increase in cell proliferation and survival (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a; Sordella et al., 2004). It is believed that inhibition of these activities with Iressa or Tarceva might contribute to a more beneficial clinical treatment outcome. However, some NSCLC patients who respond to treatment with Iressa or Tarceva lack these somatic mutations (Pao and Miller, 2005), while some patients that contain somatic EGFR mutations do not respond to treatment with Iressa (Cappuzzo et al., 2005), indicating that additional mechanism(s) exist that promote sensitivity to these drugs. Furthermore, some NSCLC patients who harbor somatic EGFR mutations in the EGFR gene and initially respond to Iressa or Tarceva eventually develop drug resistance, which coincides with the occurrence of a secondary resistance mutation T790M in the EGFR kinase domain (Kwak et al., 2005; Pao et al., 2005). *In vitro* cancer cell lines, which express EGFR harboring both the T790M mutation and a somatic activating mutation, show a 100-fold loss of sensitivity to growth inhibition by Iressa compared to the activating mutation alone (Kwak et al., 2005). More recent data indicate that irreversible inhibitors (e.g. HKI-357, HKI-272, CL-387,785) can effectively inhibit EGFR kinase activity despite the presence of the T790M mutation (Kobayashi et al., 2005; Kwak et al., 2005). The development of new

technologies that overcome limitations of current receptor tyrosine kinase screening assays is expected to lead to the discovery of novel EGFR inhibitors with broader response rate in humans and higher receptor selectivity. We utilized the bioluminescence-resonance-energy-transfer (BRET) technology and developed a new cell-based assay, which enabled us to monitor in living cells and in real time, the ligand-induced recruitment of signaling proteins (e.g., Grb2) to the EGFR. These protein interactions are key events in the assembly of larger signaling complexes, which leads to the activation of specific EGFR signaling pathways. The EGFR BRET assays have enabled a detailed and comprehensive assessment of the pharmacology and signaling properties of somatic mutations in the EGFR.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Material and Methods

Cloning and plasmids

Human cDNA's encoding EGFR, Grb2, p85, PLCγ1, Stat5A were obtained by standard RT-PCR on poly-A-RNA isolated from various human tissues or tumor cell lines. Identities of all cDNA's were confirmed by completely sequencing the open reading frames. EGFR isoforms containing somatic mutations or changes of tyrosine codons to phenaylalanine codons were generated by standard mutagenesis methods. EGFR and isoforms were in-frame subcloned into the vector pRluc-N (Perkin-Elmer, Boston, USA) to generate a chimeric cDNA expressing the EGFR-(*Renilla*)-luciferase fusion protein (EGFR-Luc). The cDNA's encoding the EGFR signaling molecules (Grb2, Stat5A, PLCγ1 and p85) were subcloned into the vector pGFP2-N or pGFP2-C (Perkin-Elmer, Boston, USA) to generate chimeric cDNA's expressing the corresponding fusion proteins: GFP2-Grb2, GFP2-p85, GFP2-PLCγ1, STAT5A-GFP2.

Drugs, compounds and antibodies

The *Renilla* luciferase substrate coelenterazine 400A (DeepBlueC) was obtained from Biotium INC. (Hayward, CA, USA). ACADIA Pharmaceuticals synthesized Tarceva and Iressa. CL-3387,785 was purchased from Calbiochem (San Diego, CA, USA). Antibodies were purchased from Chemicon Inc. (San Diego, CA, USA): Renilla luciferase antibody MAB4410; or Cell Signaling Technology, Inc. (Danvers, MA, USA): p44/42 MAP Kinase antibody 9102 and phospho-p44/42 MAP Kinase antibody 9101. Recombinant human EGF protein (100-15) was purchased from Peprotech Inc. (Rocky Hill, NJ, USA).

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

EGFR Bioluminescence resonance energy transfer (BRET) assay

HEK293T cells were cultured in DMEM (with 4500 mg/l D-glucose and glutamine, with out sodium pyruvate) (Invitrogen – GIBCO, USA), 10 % fetal bovine serum (FBS) (Hyclone, Logan, UT) supplemented with penicillin-streptomycin-glutamine solution (Invitrogen – GIBCO, USA). 10 cm plate cultures were transiently co-transfected with plasmid DNAs expressing a bioluminescence donor (1 µg plasmid DNA expressing EGFR-luciferase) and one of the following fluorescence acceptors (40 µg plasmid DNA encoding GFP2-Grb2, GFP2-p85, GFP2-PLCγ1 or Stat5A-GFP2) dependent on the signaling pathway studied. The ratio of 1:40 was predetermined in saturation experiments to be optimal for obtaining the best ligand induced increase in BRET signal. Two days after transfection, cells are harvested and resuspended in phosphate-buffered-saline pH 7.5 (PBS) with glucose and sodium pyruvate to a concentration of 2X10^6 – 4X10^6 cells/ml dependent on transfection efficiency. Transfection was performed with Polyfect (Qiagen, Valencia, CA, USA) as described by manufacturer. One day after transfection cells were serum starved for 24 hours in DMEM, 0.1 % FBS supplemented with penicillin-streptomycin-glutamine solution. Drug dilutions are prepared in Costar 3912, non-treated, white polystyrene, 96-well plates. For agonist assays, 50 µl of any drug concentration tested is incubated with 50 µl of the cell suspension for 5 minutes to establish the ligand induced recruitment of GFP2 tagged EGFR signaling proteins to the intracellular carboxy-terminus of EGFR-luciferase. For antagonist assays, 25 µl of any antagonist concentration tested is incubated for 10 minutes with 50 µl of the cell suspension followed by additional 20 minutes incubation time after addition of 25 µl of the used agonist. For both types of assay, 50 µl of *Renilla* luciferase substrate coelenterazine 400A (DeepBlueC, DBC) (5 µM final concentration) is added to activate the luciferase. Luciferase and GFP2 emissions are measured after DBC addition and a

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

1 sec shaking step for one second each. Note: GFP2 is excited through bioluminescence resonance energy transfer (BRET) between activated luciferase and GFP2, but strictly dependent on close proximity (<100 Angstrom). The time after addition of coelenterazine 400A is sufficient to reach equilibrium with luciferase activity (data not shown). Injection of DBC and recording of luminescence kinetics is automatically performed by the multiplate reader Mithras 940LB (Berthold, Bad Wildbach, Germany). The plate reader is equipped with filters to detect GFP2 emission (505-525 nm) and *Renilla* luciferase emission (375-445 nM). The BRET signal is calculated as the ratio between the *Renilla* luciferase emission and the GFP2 emission corrected by the background emissions of non-transfected cells. The first 5 minutes of the time course of EGF induced BRET-2 signal increase in the EGFR/Grb2 BRET-2 assay (Figure 1B) was generated by a timely coordinated injection of EGF and DBC, performed by the multiplate reader Mithras 940LB (Berthold, Bad Wildbach, Germany). Dose response curves and non-linear regression analysis are performed with the software PRISM (GraphPad software INC, CA, USA) to obtain IC_{50} and EC_{50} values.

Cell extract formation and Western blot

Transfected BRET assay cells were harvested and resuspended in D-PBS (2X10^6 – 4X10^6 cells/ml dependent on transfection efficiency). Whole cell extracts were prepared after incubation with 25 nM EGF prepared in D-PBS or with D-PBS alone in RIPA buffer (including proteinase inhibitor cocktail). Samples were solubilized in loading buffer and directly separated on 12% SDS-PAGE. Western Blot analysis was performed as recommended for the antibodies purchased from Cell Signaling Technology, Inc., MA,USA.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Signaling pathway specific EGFR bioluminescence resonance energy transfer (BRET-2) assays

The rapid ligand-stimulated autophosphorylation of specific tyrosine residues in the intracellular carboxy-terminus of receptor tyrosine kinases (RTKs) is an obligatory event in RTK signaling (Schlessinger, 2000; Schlessinger, 2002). The phosphorylated tyrosine residues serve as docking sites for a diverse set of proteins, which are involved in building, shaping and directing the specific RTK downstream signaling pathways (Schlessinger, 2000; Schlessinger, 2002). Traditionally, RTK pharmacology and signaling have been quantitatively studied using mainly immunological methods that detect RTK phosphorylation (Olive, 2004) or downstream effects on proliferation. We designed a functional receptor tyrosine kinase (RTK) assay that utilizes the bioluminescence-resonance-energy-transfer (BRET-2) technology (Gales et al., 2005). We applied this technology to quantitatively monitor, in living cells, the recruitment of various EGFR signaling proteins, which interact with activated EGFR to link the receptor to the four main RTK signaling pathways. The various signaling proteins we studied include: The adapter proteins Grb2 and Shc (MAP-kinase proliferation pathway), Stat5A (STAT pathway), phospholipase Cγ1 (PLCγ1 – PKC/calcium pathway), and p85, the regulatory subunit of phosphatidyl-inositol-3-kinase (PI3K-Akt survival pathway). The human EGFR protein was in frame carboxy-terminal tagged with *Renilla* luciferase (Luc), while Green Fluorescence Protein isoform 2 (GFP2) was fused in-frame to the amino-termini of Grb2, PLCγ1 and p85 and the carboxy-terminus of Stat5A (see materials and methods). To perform an EGFR BRET assay, EGFR-luciferase (EGFR-Luc) and a GFP2-tagged signaling protein were transiently co-expressed in HEK293T. Cells were incubated for 20 minutes with variable concentrations of epidermal growth factor (EGF) to activate EGFR and stimulate recruitment of the GFP2-tagged

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

signaling molecule to EGFR-Luc. BRET-2 signals were measured by detecting luciferase and GFP2 emissions, after addition of the cell-permeable *Renilla* luciferase substrate coelenterazine 400A (DeepBlueC), and calculating the ratio between the detected GFP2 and Luc emissions (see material and methods). The Bioluminescence-resonance-energy-transfer (BRET) between activated luciferase (luminescence donor) and GFP2 (fluorescence acceptor) causes excitation of GFP2, but this signal is strictly dependent on the proximity of both proteins, so that the BRET-2 signal in this assay directly correlates with the activation of EGFR and recruitment of GFP2-tagged EGFR effector proteins. A recent report showed that the BRET technology can also be applied to monitor in living cells the recruitment of insulin receptor substrate-1 (IRS-1) or protein tyrosine phosphatase-1B (PTP-1B) to the insulin receptor (Laursen and Oxvig, 2005).

Figure 1 shows agonist and antagonist dose response curves in cells co-transfected with wild type EGFR-Luc and GFP2-Grb2. Application of variable concentrations of the potent agonist EGF caused a dose dependent BRET-2 signal increase in the wild type EGFR BRET-2 assay (pEC_{50} = 10.1, Figure 1A), demonstrating the high sensitivity of the assay. Already 10 seconds after applying a high dose of EGF (17 nM), the EGFR BRET-2 signal reached around 90% of the maximal response (time course in Figure 1B). The EGF induced BRET-2 signal peaked by 5 minutes and persisted for more than 20 minutes (Figure 1B and data not shown). The observed increase in the Grb2/BRET-2 signals was dependent on phosphorylation of multiple EGFR tyrosine residues in the intracellular carboxy-terminus. Changing the tyrosine (Y) residues 1068, 1086, 1101, 1148 and 1173 to phenylalanine (F) in EGFR-Luc resulted in 70% +/- 0.6% reduction of the EGF induced BRET-2 signal in the Grb2/BRET-2 assay (EGFR wild type: 0.23 +/- 0.006 (-EGF), 0.60 +/- 0.006 (+ EGF) versus EGFR quintuple mutant EGFR isoform: 0.19

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

+/- 0.002 (-EGF), 0.32 +/- 0.002 (+ EGF), and data not shown). A mutant EGFR isoform carrying the Y1086F, Y1101F, Y1114F and Y1173 mutations showed only a 45% reduction of the EGF induced Grb2/BRET-2 signal (data not shown). At least 6 phosphorylated tyrosine residues have been identified in the carboxy-terminus of EGFR that mediate multiple direct or indirect interactions of the adapter protein Grb2 with EGFR (Schulze et al., 2005). Our results are consistent with previous results that demonstrate that the recruitment of EGFR adapter proteins is dependent on tyrosine phosphorylation (Schlessinger, 2000; Schlessinger, 2002). The EGF stimulated recruitment of the GFP2-tagged signaling proteins to EGFR-Luc could be effectively inhibited through the co-application of various EGFR kinase domain inhibitors (e.g., EGFR Grb2/BRET-2 assay in Figure 1C: AG1478, pIC_{50} = 8.38 (5 nM); PD168393, pIC_{50} = 8.21 (6.3 nM); Iressa, pIC_{50}= 7.33 (46 nM); Tarceva, pIC_{50} = 7.73 (19 nM) or neutralizing EGFR antibodies (data not shown). In contrast, incubation of the cells with varying concentrations of the kinase domain inhibitor Iressa in the absence of exogenous EGF slightly reduced the BRET-2 signal, suggesting the presence of constitutive activity in wild type EGFR (Figure 2A, Iressa, open circles). The BRET-2 signal was reduced to around 0.19 for wild type at the highest concentrations of Iressa, which is likely to be the baseline BRET-2 signal for the EGFR/Grb2 BRET-2 assay. In contrast to EGF stimulated EGFR activity, the constitutive activity of the wild type EGFR could not be neutralized with anti-human EGF antibodies (data not shown).

EGFR has previously been carboxy-terminally tagged with GFP isoforms which did not change half-life, ligand-induced tyrosine phosphorylation, or internalization properties (Hayes et al., 2004). Interaction of EGFR and Grb2 in living cells has also been visualized by fluorescence resonance energy transfer (FRET) microscopy ((Sorkin et al., 2000). Additionally, EGFR

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

proteins have been shown to recruit YFP tagged Grb2 or GFP tagged PLCγ1 from the cytoplasm to the plasma membrane (Hayes et al., 2004; Sorkin et al., 2000). Our wild type EGFR-luciferase chimera did show pharmacological and signaling properties similar to reported data for untagged EGFR receptors (Fry et al., 1998; Hynes and Lane, 2005), suggesting that the luciferase tag did not severely alter the function of EGFR.

The activation of EGFR signaling pathways is controlled by the recruitment of specific signaling molecules to phosphorylated tyrosine residues in EGFR domains of the intracellular carboxy-terminus. The EGFR BRET-2 experiments described in Figure 1 were focusing on the recruitment of Grb2, an adapter protein that transduce EGFR activity into the MAP kinase pathway. We also studied wild type EGFR pharmacology in BRET-2 assays using the signaling molecules p85, PLCγ1 or Stat5A (Table 1). All wild type EGFR BRET-2 assays showed a high sensitivity to the agonist EGF (mean pEC_{50} = 10.1 +/- 0.11, table 1). These results are in close agreement with previous methods for measuring wild type EGFR activity, thereby validating the EGFR BRET-2 assay. Thus, we used these EGFR BRET-2 assays to study the pharmacology and signaling properties of somatic EGFR mutations in lung cancer and in particular compared the pharmacological activities of the EGFR inhibitors Iressa and Tarceva.

Somatic mutations in EGFR cause constitutive activity and affect responsiveness to EGF

Somatic EGFR mutations have been identified in NSCLC, which activate EGFR signaling (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). We studied four somatic EGFR mutations in the EGFR BRET-2 assays: L858R, the most common point mutation observed in NSCLC patients (exon 21) is localized in the activation loop of the EGFR TK domain; G719C

(exon 18); localized in the nucleotide phosphate binding loop (P-loop), and the deletion mutations Δ752-759 and Δ747-749 A750P (exon 19), both localized close to the ATP binding region. EGFR-Luc isoforms carrying these mutations were co-transfected with GFP2-Grb2 to evaluate the effects of these somatic mutations on MAP-kinase pathway signaling. The presence of the described mutations in EGFR-Luc did not significantly affect the expression levels compared to wild type EGFR-Luc (data not shown and figure 5 bottom). In the absence of exogenously added EGF, we observed significant constitutive activity for all four mutations tested (compare no ligand baselines in Figure 2A-E), which is reflected by the higher Grb2/BRET-2 assay signal of the mutants compared to the wild type EGFR. While wild type EGFR exhibited a BRET-2 signal of 0.21 in the absence of EGF, the L858R mutant receptor showed the highest level of constitutive activity with a BRET-2 signal of 0.33. All constitutive activities were efficiently inhibited both by Iressa (Figure 2A-E) and Tarceva (data not shown and table 1). The P-loop G719C mutation (exon 18) had the lowest constitutive activity of the four mutations tested, while the three other mutants localize in two mutation hotspots in exons 19 and 21 with the highest constitutive activity. Our results indicate that the somatic mutations tested cause an increase in the constitutive interaction between mutant EGFR-Luc isoforms and GFP2-Grb2. Iressa and Tarceva effectively inhibit this increase in the BRET-2 signal, probably by competing with ATP binding at the intracellular catalytic tyrosine kinase domain. Similar results were obtained in the EGFR/Shc BRET-2 assay, which monitored the recruitment of the adapter protein Shc42 to EGFR-Luc (data not shown). Our results suggest that the studied somatic mutations in EGFR cause a constitutive increase in MAP-kinase pathway signaling (see Figure 5 and discussed in later section).

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Importantly, treating the various mutant EGFR isoforms with EGF revealed dramatic differences in their respective EGF responsiveness. EGF is a very potent agonist for wild type EGFR (Table 1, pEC_{50} = 10.1, Figures 1A and 2A, filled circles) in the EGFR/Grb2 BRET-2 assay, demonstrating the sensitivity of the EGFR BRET-2 assay. While all mutant EGFR isoforms are only slightly less potent in responding to EGF, they show more dramatic differences in efficacy compared to wild type EGFR (Figure 2A-E, filled symbols, and table 1). For the wild type receptor, the BRET-2 signal increased from 0.21 in the absence of EGF to 0.55 in the presence of the maximal effective dose of EGF (Figure 2A, filled circles). However, for the EGFR point mutants, the signal with EGF increased to only 0.50 for G719C and 0.45 for L858R (Figure 2B and C, filled symbols, respectively). The EGF signal was further impaired in the deletion mutants, which showed only a slight ligand induced increase in the BRET-2 signal to 0.35 (Figure 2D-E, filled symbols), indicating a strong impairment in transducing EGF signals into the MAP-kinase pathway signaling. Therefore, none of the tested constitutively active EGFR mutants reached wild type EGFR activity level after EGF stimulation. This reduced response to EGF has previously not been recognized because other methods did not clearly separate constitutive activity from EGF stimulated responses (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). We could show that somatic mutation in EGFR dramatically increase the level of constitutive receptor activity and decrease the responsiveness to EGF. These results demonstrate that EGFR BRET assays are a useful pharmacological tool that allows the separate study of ligand-dependent and ligand-independent EGFR function.

Iressa and Tarceva effectively inhibit constitutive activity of EGFR isoforms

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

While the constitutive activity of wild type EGFR in the EGFR/Grb2 BRET-2 assay is inhibited by Iressa at a pIC_{50} of 6.59 (Figure 2A, open circles, and Table 1), the constitutive activities of the somatic mutants as measured by the same assay are 5-10 fold more sensitive to inhibition by Iressa (Figure 2 B-D, open symbols), with pIC_{50} values ranging from 7.41-7.59 (Table 1). Tarceva produced similar results (Table 1). These results are consistent with previous findings in cell proliferation assays of cancer cell lines endogenously expressing EGFR mutants (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004b; Sordella et al., 2004), and provides one possible explanation for the high clinical responsiveness of NSCLC patients who harbor somatic EGFR mutations and are treated with these EGFR inhibitors. No significant differences were observed between Iressa and Tarceva acting at the various EGFR mutants, except that Tarceva appears slightly more potent in inhibiting the wild type and mutant EGFR isoforms (ΔIC_{50} = 0.41 +/- 0.14 for EGFR/Grb2 BRET-2 assays). It is surprising that all of the constitutively active mutations increase inhibitor potency. In many other receptor systems, constitutively active mutations decrease the potency of inhibitors. This may reflect fundamental differences in the receptor interaction with signal transduction inhibitors (e.g. Iressa and Tarceva) versus classical receptor antagonists and inverse agonists (Spalding et al., 1995).

Constitutive activity of EGFR isoforms is predominantly transduced through the PI3K/Akt survival pathway

Based on the results from the EGFR/Grb2 BRET-2 assay, we decided to study the effect of the L858R and the Δ752-759 mutations on other EGFR signaling pathways. The results of all experiments are summarized in Table 1 and presented in Figure 3. For each receptor isoform and signaling pathway studied, the BRET-2 signals are transformed in % and normalized to the wild

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

type EGFR response. The 100% BRET-2 signal of wild type EGFR represents the sum of EGF stimulated wild type EGFR activity plus the constitutive activity of wild type EGFR (determined by Iressa inhibition and indicated as a filled bar for wild type (WT) EGFR in figure 3A-E). With the exception of the Stat5A effector, EGF as agonist showed a slight loss of potency for activating the two mutants EGFR isoforms compared to the wild type EGFR (Table 1). Consistent with the EGFR/Grb2 BRET-2 assay (Figure 2 and Figure 3A), both mutant EGFR isoforms (L858R and Δ752-759) show constitutive activity (in the absence of EGF) for all pathways (Figure 3A-D open bars), but with quantitative differences. Importantly, all EGFR mutants tested predominantly signal through the PI3K/Akt survival pathway (Figure 3C). For the L858R mutant, the constitutive activity (Figure 3C, L858R open bar) is about 70% of the total wild type response (Figure 3C, WT filled bar). This observation correlates well with previous studies that detected increased Akt phosphorylation in the cancer cell line H-1975 that endogenously express EGFR L858R (Sordella et al., 2004). Meanwhile, the corresponding constitutive signaling activity of the L858R mutant through the MAP-kinase, STAT and PLCγ1-calcium signaling pathways ranged only between 28% and 40% of the total wild type responses (L858R in Figure 3A, B and D, open bars). The deletion mutant EGFR Δ752-759 showed a similar profile for constitutive activity and coupling to the different signaling pathways, with 54% activity in the PI3K/Akt pathway (Δ752-759 in Figure 3C, open bar) and lower levels of activity 30-35% in the other pathways (Δ752-759 in Figure 3A, B, and D, open bar). It will be interesting to determine in the future with the EGFR/p85 BRET-2 assay if all other described EGFR mutants show high constitutive activation of the PI3K/Akt pathway and if this activity directly correlates with increased drug sensitivity. A recent study used an EGF independent Ba/F3 cell transformation assay to show constitutive activity of the EGFR L858R and G719S

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

isoforms and a differential sensitivity of both isoforms to small molecule inhibitors (Jiang et al., 2005). We also found differences in the EGF responsiveness between the L858R and Δ752-759 mutants. When the L858R isoform was treated with EGF in BRET assays monitoring STAT, PI3K/Akt or PLCγ1-calcium signaling, we detected in all assays a slightly reduced EGF responsiveness compared to wild type EGFR (Figure 3B-D, compare differences between open and filled bars). This result was similar to the slightly reduced EGF responsiveness of EGFR L858R detected in the Grb2/BRE-2 assay (Figure 2C and Figure 3A). Interestingly, the deletion mutant EGFR Δ752-759 showed a large quantitative difference in the EGF stimulated response, when comparing the Stat5A/BRET-2 and Grb2/BRET-2 assays (compare difference between open and filled bars for Δ752-759 in Figure 3A and Figure 3B). EGF stimulated Stat5A-GFP2 recruitment to EGFR-Luc Δ752-759, but did not significantly stimulate the recruitment of GFP2-Grb2. It is possible that EGFR receptors, which carry this mutation, are impaired in transducing the EGF signal downstream into the MAP-kinase pathway, but are still able to activate the STAT pathway. These results suggest that a specific somatic EGFR kinase domain mutation can differentially affect EGFR signaling pathways.

In contrast to the differential constitutive activity and EGF responsiveness of the mutant EGFR isoforms, the EGFR inhibitors Iressa or Tarceva do not show a preference to inhibit the constitutive activity of a specific mutant EGFR isoform (table 1). However, Iressa and Tarceva are in general more potent in inhibiting the constitutive activity of mutant EGFR isoforms than of the wild type EGFR. This observation is consistent with the increase in drug sensitivity to inhibit proliferation of cancer cell lines that harbor these mutations compared to cancer cell lines that express only wild type EGFR (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a;

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Sordella et al., 2004). We could not detect constitutive activity in the wild type EGFR with the EGFR/Stat5a or EGFR/PLCγ1 BRET-2 assays, which prevented us to quantitate the increase of drug sensitivity. Since our results are derived from heterologous EGFR expression in HEK293T cells, it suggests that the presence of somatic mutations in EGFR is principally sufficient to increase drug sensitivity of cancer cells that express mutant EGFR isoforms.

Impact of T790M mutation on inhibition of constitutive EGFR activity by Tarceva and Iressa

Many lung cancer patients will relapse and acquire drug resistance to both Iressa and Tarceva during their treatment regiment. Acquisition of drug resistance is a complex process involving multiple poorly characterized pathways, one involving the occurrence of resistance mutations (Dean et al., 2005). Recently, several studies reported the identification of an acquired secondary resistance mutation (T790M) in the EGFR kinase domain of patients, who were treated and initially responded to Iressa and Tarceva (Kobayashi et al., 2005; Kwak et al., 2005; Pao et al., 2005). The T790M mutation was only found in the presence of an activating EGFR mutation in tumor samples, although only in a small fraction of the cells within the entire tumor. The mutation has also been found in patients that did not undergo treatment with Iressa and Tarceva. Interestingly, a recent report identified T790M as a inherited germline EGFR mutation potentially associated with susceptibility to late onset NSCLC (Bell et al., 2005).

We analyzed a mutant EGFR variant bearing the T790M mutation alone and mutant EGFRs that carry T790M in combination with the L858R or Δ747-749 A750P mutations in the EGFR/p85 BRET-2 assay. Our results show that the T790M mutation alone generates a highly constititutive active EGFR receptor (Figure 3E open bar). Additionally, we analyzed the double mutant

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

EGFRs L858R T790M and Δ747-749 A750P T790M, both reported to occur in patients that developed drug resistance in NSCLC (Kobayashi et al., 2005; Kwak et al., 2005; Pao et al., 2005). Both double mutants showed the highest levels of constitutive activity of all mutants tested in this report. The constitutive activity of the EGFR L858R T790M receptor (Figure 3E, open bar and Figure 4A, open circle) reached the same maximal activity level as the wild type EGFR treated with EGF, while the constitutive activity level of EGFR Δ747-749 A750P T790M reached 80% of the maximal EGF response for wild type EGFR (Figure 3E, open bar) similar to the constitutive activity of the T790M mutation alone. Meanwhile, the EGF responsiveness of both double mutants was nearly completely impaired (Figure 3E, compare differences between open and filled bars and Figure 4A). Our results strongly indicate that the development of drug resistant cells carrying the T790M mutation is accompanied by a dramatic increase in constitutive activation of the PI3K/Akt pathway (Figure 3E and 4A). We found similar robust constitutive activation of the MAP-kinase pathway by the double mutant receptors (data not shown).

We next analyzed the effect of Iressa and Tarceva on EGFR isoforms carrying the T790M mutation. Tarceva was not effective in inhibiting the constitutive activity of the EGFR T790M isoform (Figure 4B, closed circles). However, despite similarities with Tarceva in structure and mode of action, Iressa inhibited the constitutive activity of the T790M isoform (85% inhibition with 33 μM high dose), but with a lower potency compared to the other somatic mutants (pIC_{50} = 5.3 +/- 0.033) (Figure 4B closed triangles). In contrast to the prediction from the T790M mutation alone, we observed significant but partial inhibition (50-75%) of the double mutants by Iressa or Tarceva (Figure 4C and 4D). These results are in good agreement with experiments in

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

which the proliferation of the cancer cell line NCI-H1975, which express the double mutant EGFR L858R T790M isoform, is significantly suppressed by high concentration of Iressa. Immunoblot data suggest that EGFR tyrosine 1068 and Akt phosphorylation is suppressed in this cell line at high micromolar concentrations of Iressa (Kobayashi et al., 2005). Interestingly, Tarceva was slightly more potent in inhibiting EGFR L858R T790M activity than Iressa in our BRET/p85 BRET-2 assay (Tarceva pIC_{50} = 5.89 +/- 0.06 vs. Iressa pIC_{50} = 5.25 +/- 0.07). A similar potency difference was observed for the deletion mutant EGFR Δ747-749 A750P T790M (Tarceva pIC_{50} = 6.33 vs. Iressa pIC_{50} = 5.80). This analysis of the EGFR T790M mutation in the EGFR BRET-2 assay correlates well with the known resistance of T790M-bearing tumors to Iressa and Tarceva and show that it is important to develop new strategies for inhibiting the drug resistant cell clones because of their more aggressive properties. Recent studies demonstrate that irreversible EGFR inhibitors are more effective in inhibiting the constitutive activity of these EGFR double mutants (Carter et al., 2005; Kobayashi et al., 2005; Kwak et al., 2005).

We tested the irreversible inhibitor CL-387,785 in the EGFR/p85 BRET-2 assay with EGFR L858 T790M and EGFR Δ747-749 A750P T790M and observed complete inhibition of constitutive activity with an pIC_{50} of 6.86 +/- 0.14 and 6.81 +/- 0.12, respectively (and data not shown). Wild type EGFR showed a higher sensitivity for inhibition with a pIC_{50} of 9.4 +/- 1.1. Similar results were obtained in the EGFR/Grb2 assay (data not shown). Very early treatment of primary tumors with irreversible inhibitors might reduce the risk to develop T790M related drug resistance and relapses. In addition, the lack of EGF responsiveness and the decreased sensitivity for the EGFR inhibitor Iressa and Tarceva suggests that concurrent EGF treatment could rescue patients from toxicities of EGFR inhibitors that would not be tolerated otherwise.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Mutant EGFR isoforms constitutively activate downstream ERK1/2 in the EGFR/Grb2 BRET-2 assay

We explored how the signaling through the MAP-kinase pathway downstream of the adapter protein Grb2 is affected by somatic EGFR mutations in our EGFR/Grb2 BRET-2 assays. ERK1/2 are the prototypic MAP-kinases and are activated downstream of Grb2. We studied ERK1/2 protein expression and phosphorylation in BRET-2/Grb2 assay cells by western blotting (Figure 5). Cells expressing GFP2-Grb2 in combination with the wild-type EGFR-Luc, the mutated EGFR-Luc isoform L858R, and the mutated EGFR-Luc isoform L858R T790M were either left untreated or treated with EGF and then analyzed. We found similar levels of EGF induced (Thr202/Tyr204) phosphorylation of ERK1/2 (molecular weight 42 and 44 kDa) in all BRET-2 samples analyzed (Figure 5, lanes 2, 4, 6, and 8) including the no receptor control (Figure 5, lane 2). HEK293T cells endogenously express a low level of EGFR, which is sufficient for ERK1/2 activation in non-transfected cells (Figure 5, lane 2). Introduction of the wild-type EGFR-Luc or of the two mutant EGFR isoforms did not enhance the EGF-induced phosphorylation of ERK1/2, which are detected as 42/44 kDa protein bands by western blotting (Figure 5, compare lanes 2,4,6 and 8). However, our blots additionally detected an increase of phosphorylated ERK1/2 proteins in the form of a reduced-mobility band (Figure 5, lanes 3-8), which was absent in cells lacking transfected EGFR-Luc isoforms (Figure 5, lane 2). Western blotting using an antibody against *Renilla* luciferase shows that the reduced-mobility phospho-EEK1/2 bands migrate at the same blot location as the EGFR-Luc isoforms (Figure 5 lower panel). These observation suggest the formation of a complex between phosphorylated ERK1/2 and activated EGFR, and is consistent with the results of Habit et al. (2003), who demonstrated

that the over-expression of EGFR in HEK293 cells or cancer cell lines (e.g., A431) leads to formation of a stable complex between EGFR and phosphorylated ERK1/2 (Habib et al., 2003). We could not detect this complex using the ERK1/2 protein antibody in western blotting, possibly due to masking the ERK1/2 antibody epitope in the complex (Figure 5 middle). Importantly, the observed EGFR-Luc-phospho-ERK1/2 complex formation was enhanced by treatment with EGF, particularly in the wild-type EGFR-Luc transfected cells (Figure 5, compare lanes 7 and 8). Furthermore, the EGFR L858R and L858R T790M mutants, which show constitutive activity in the BRET-2/Grb2 assays (Figures 2-4), showed increased amounts of this reduced-mobility phospho-ERK1/2 complex compared to wild-type EGFR in the absence of EGF (Figure 5, compare lanes 3 and 5 with lane 7 in top panel). For the double mutant L858R T790M, the amount of this complex could not be further enhanced with EGF (Figure 5, lanes 3 and 4), consistent with the maximal constitutive activation observed with this mutant in the BRET-2/Grb2 assay (Figures 3E). Our result are consistent with a recent report demonstrating increased MAP-kinase signaling by showing constitutive phosphorylation of the adapter protein Shc in cells expressing EGFR L858R (Greulich et al., 2005). In contrast Sordella et.al, (2004) showed that the somatic EGFR mutation L858R does not affect ERK1/2 phosphorylation despite an increase in autophosphorylation of tyrosine residue 1068, which is involved in Grb2 binding (Sordella et al., 2004). Overall, the observed activation of downstream MAPK signaling events in the form of an activated EGFR-ERK1/2 complex correlates well with the pharmacological data collected with our BRET-2 assays. Overexpression of EGFR is commonly observed in various cancer types and is involved in cell transformation and cancer progression (Pao and Miller, 2005). Understanding the pharmacology and signaling properties of over-expressed

mutated EGFR isoforms might help to obtain a better prognosis about the disease outcome and improve the decision about treatment options.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

We developed a quantitative bioluminescence-resonance-energy-transfer (BRET-2) assay that allowed us to effectively study the pharmacology and signaling properties of somatic mutations in EGFR. Although our EGFR BRET assays are based on the heterologous over-expression of luciferase tagged EGFR isoforms as a luminescence donor and GFP2-tagged adapter proteins as fluorescence acceptors (e.g., GFP2-Grb2) in HEK293T cells, they proved to be valuable in assessing the pharmacological properties of EGFR agonists, antagonists and to determine the level of constitutive activity. It is possible that tagging EGFR and the signaling proteins alters their protein conformations and influences their interactions. However, it appeared not to prevent efficient EGF induced recruitment of the adapter proteins to EGFR in our BRET assays (Figures 1 and 2, table 1). It is important to note that all adapter proteins used in our BRET-2 assays were full-length protein moieties. Importantly, despite the use of tagged proteins in the EGFR Grb2/BRET-2 assay, we were able to detect downstream modulation of ERK1/2 protein kinases in the MAP-kinase pathway (Figure 5). The level of constitutive activity from different mutant EGFR isoforms was mirrored in the levels of ERK1/2 phosphorylation (Figure 5). In this specific case, over-expression of luciferase tagged EGFR also caused a ligand-induced interaction of phosphorylated ERK1/2 kinases with EGFR-Luc (Figure 5), which has been previously observed in cancer cell lines (Habib et al., 2003). Therefore, our EGFR BRET-2/Grb2 assays let us study both EGFR pharmacology and EGFR-Erk1/2 complex formation side by side in one system and will also facilitate research to understand the role of this complex in cancer. The results from our BRET assays are consistent with published data from other studies, suggesting that monitoring the recruitment of EGFR signaling proteins by BRET is a useful tool to analyze EGFR signaling.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Besides the demonstration of EGFR BRET assays as a pharmacological tool, our results reveal important new insights into the pharmacology and signaling properties of somatic EGFR mutations in lung cancer. As previously reported, our results also show that somatic mutations in EGFR are sufficient to increase constitutive activity and enhance the sensitivity to the EGFR inhibitors Iressa or Tarceva. However, in contrast to recent studies, which detected constitutive activity of mutated EGFR isoforms in growth or foci formation assays (Greulich et al., 2005), we were able to analyze constitutive EGFR activity in selected signaling pathways. In particular, our BRET assays detected a preferentially constitutive activation of the PI3K/Akt-survival pathway for all mutant EGFR isoforms tested. These results are consistent with the previously reported strong activation of the PI3K pathway in cancer cell lines, which express these mutated EGFR isoforms (Pao and Miller, 2005). However, these earlier studies could not distinguish between ligand dependent and independent activation. Importantly, we show that the level of constitutive EGFR activity and the response of EGFR isoforms to EGF are differentially impacted by these mutations. The impairment of EGF responsiveness of mutated EGFR isoforms has previously not been recognized. In particular, the deletion mutations (e.g., Δ752-759) appear to weaken the EGF responsiveness of the MAP-kinase signaling pathway more severely than somatic mutations that only change single amino acids (e.g., L858R). The severe loss of EGF responsiveness in the deletion mutants might interfere with the function of the autocrine EGF-EGFR loop that is known to contribute to cancer cell growth and maturation (Tateishi et al., 1990). Interestingly, the Δ752-759 mutant showed only a partial impairment in EGF responsiveness in the STAT pathway, in contrary to results obtained for the MAP-kinase pathway. This disparity may be caused by a structural change in the tyrosine kinase domain, which differentially affects the EGF-stimulated autophosphorylation at specific tyrosine residues and hence differentially affects

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

the recruitment of Grb2 and Stat5A to the EGF-activated receptors. Thus, somatic EGFR mutations in lung cancer cannot be viewed as identical with respect to their EGF responsiveness. This finding could have important clinical implications for disease prognosis and drug response of individual NSCLC patients.

It is currently unclear why the EGFR inhibitors Iressa and Tarceva, which are believed to share the same mechanism of action, show differences in the clinical efficacy affecting overall survival in NSCLC (Tyagi, 2005). We extensively compared the pharmacology for both drugs acting on the wild type and mutant EGFR isoforms in all four major EGFR signaling pathways using the EGFR BRET assays. No comprehensive dataset has previously been reported that compared the pharmacology of Iressa and Tarceva acting on several mutated EGFR isoforms and the main EGFR signaling pathways. Both drugs showed very similar pharmacological properties, except that Tarceva is slightly more potent than Iressa in inhibiting EGFR signaling pathways (see Table 1). The effective steady state plasma concentrations reported for Iressa (0.4 – 1.4 μM) and Tarceva (3 μM) reached in the clinic (Baselga et al., 2002; Hidalgo et al., 2001) are significantly higher than required to inhibit EGF stimulated EGFR signaling *in vitro* BRET-2 assays. The results from our BRET-2 assays imply that both drugs would saturate mutant EGFR isoforms and inhibit their constitutive activity during treatment if these concentrations were reached in tumors. Importantly, skin rash and gastrointestinal side effects appear more commonly with Tarceva than with Iressa. The development of skin rash is dose-dependent and seems to be correlated to the clinical response and survival, thus making rash a potential surrogate marker of activity (Perez-Soler et al., 2005). Therefore, it might be possible that clinical doses of Iressa do

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

not always saturate EGFR in the skin and maybe also in the tumor tissue to explain the difference in clinical efficacy of both drugs.

Acquisition of resistance to the treatment with Iressa or Tarceva has been observed in the clinical treatment of NSCLC. In some patients the occurrence of resistance has been correlated with the presence of the secondary resistance mutation T790M in EGFR. The T790M mutation has recently also been found in the germline of lung cancer families (Bell et al., 2005). Structural models of the EGFR kinase domain bearing the T790M mutation predict a steric hindrance for Tarceva or Iressa binding to the ATP binding site (Kobayashi et al., 2005). We analyzed the pharmacological properties of the T790M mutation alone and the T790M mutation in combination with primary activating mutations with our EGFR BRET-2 assays. Our data reveal high levels of constitutive activity and EGF responsiveness for the mutant EGFR isoform T790M (T790M in Figure 3B, open bar). Carrying this mutation as the only EGFR mutation in the germ line might be sufficient to promote lung cancer, which would explain the co-segregation of lung cancer and the presence of this mutation in an extended family (Bell et al., 2005). The occurrence of the T790M mutation in the background of activated mutant EGFR isoforms (e.g., L858R or Δ747-749 A750P) has been correlated with the development of drug resistance to Iressa or Tarceva and the recurrence of tumor tissue in NSCLC. Both double mutant EGFR isoforms: L858R T790M and Δ747-749 A750P T790M are highly constitutively active and do not respond to EGF (Figure 3E). In particular, the constitutive activity of EGFR L858R T790M reached the level of a fully EGF-stimulated wild type EGFR. We speculate that cells expressing EGFR L858R T790M might be very aggressive cancer cells, which are drug resistant and less dependent on EGF, and therefore might give rise to a less favorable disease

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

prognosis during relapse. The development of new EGFR inhibitors that efficiently target the T790M containing receptor isoforms might facilitate the development of new effective drugs that will reduce the risk of drug resistance and relapse. It might be important to administrate these drugs already during treatment of the primary tumor, before a potential relapse occurs. It is important to note, that the IC_{50} of inhibiting the double mutant EGFRs in our *in vitro* cell based BRET-2 assay is similar to the effective concentrations of Tarceva and Iressa reported in the human plasma of treated NSCLC patients (Baselga et al., 2002; Hidalgo et al., 2001). Therefore, small differences in the plasma concentrations of Iressa or Tarceva could cause significant changes in their efficacy to inhibit the EGFR PI3K/Akt pathway in the double mutants. The lack of complete inhibition might explain the acquisition of drug resistant cells. Large differences in human plasma concentrations for Iressa or Tarceva have been reported between different NSCLC patients (Baselga et al., 2002; Hidalgo et al., 2001). However, occurrence of the T790M mutation in activated cancer cell lines reflects only one of several mechanisms of acquiring drug resistance (Dean et al., 2005). It is completely unknown when and how the T790M mutation arises.

The results from our study demonstrate that EGFR BRET assays are a powerful tool to study pharmacology and signaling properties of EGFR. Due to the similar mechanism of activation and signal-transduction between EGFR and other RTK's, the BRET technology should be adaptable to study pharmacology and signaling properties of the whole RTK family.

Acknowledgments

The authors like to thank Andria Lee Del Tredici for critical reading of the manuscript.

References

Baselga J, Rischin D, Ranson M, Calvert H, Raymond E, Kieback DG, Kaye SB, Gianni L, Harris A, Bjork T, Averbuch SD, Feyereislova A, Swaisland H, Rojo F and Albanell J (2002) Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J Clin Oncol* **20**:4292-4302.

Bell DW, Gore I, Okimoto RA, Godin-Heymann N, Sordella R, Mulloy R, Sharma SV, Brannigan BW, Mohapatra G, Settleman J and Haber DA (2005) Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. *Nat Genet* **37**:1315-1316.

Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA, Jr. and Varella-Garcia M (2005) Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* **97**:643-655.

Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, Milanov ZV, Atteridge CE, Biggs WH, 3rd, Edeen PT, Floyd M, Ford JM, Grotzfeld RM, Herrgard S, Insko DE, Mehta SA, Patel HK, Pao W, Sawyers CL, Varmus H, Zarrinkar PP and Lockhart DJ (2005) Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. *Proc Natl Acad Sci U S A* **102**:11011-11016

Dean M, Fojo T and Bates S (2005) Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**:275-284.

Fry DW, Bridges AJ, Denny WA, Doherty A, Greis KD, Hicks JL, Hook KE, Keller PR, Leopold WR, Loo JA, McNamara DJ, Nelson JM, Sherwood V, Smaill JB, Trumpp-Kallmeyer S and Dobrusin EM (1998) Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. *Proc Natl Acad Sci U S A* **95**:12022-12027.

Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP and Baselga J (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* **21**:2237-2246.

Gales C, Rebois RV, Hogue M, Trieu P, Breit A, Hebert TE and Bouvier M (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods* **2**:177-184.

Goldberg RM (2005) Cetuximab. *Nat Rev Drug Discov* **Suppl**:S10-11.

Greulich H, Chen TH, Feng W, Janne PA, Alvarez JV, Zappaterra M, Bulmer SE, Frank DA, Hahn WC, Sellers WR and Meyerson M (2005) Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. *PLoS Med* **2**:e313.

Habib AA, Chun SJ, Neel BG and Vartanian T (2003) Increased expression of epidermal growth factor receptor induces sequestration of extracellular signal-related kinases and selective attenuation of specific epidermal growth factor-mediated signal transduction pathways. *Mol Cancer Res* **1**:219-233.

Hayes N, Howard-Cofield E and Gullick W (2004) Green fluorescent protein as a tool to study epidermal growth factor receptor function. *Cancer Lett* **206**:129-135.

Herbst RS, Fukuoka M and Baselga J (2004) Gefitinib--a novel targeted approach to treating cancer. *Nat Rev Cancer* **4**:956-965.

Hidalgo M, Siu LL, Nemunaitis J, Rizzo J, Hammond LA, Takimoto C, Eckhardt SG, Tolcher A, Britten CD, Denis L, Ferrante K, Von Hoff DD, Silberman S and Rowinsky EK (2001) Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J Clin Oncol* **19**:3267-3279.

Hynes NE and Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* **5**:341-354.

Jiang J, Greulich H, Janne PA, Sellers WR, Meyerson M and Griffin JD (2005) Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. *Cancer Res* **65**:8968-8974.

Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG and Halmos B (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352**:786-792.

Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, Harris PL, Driscoll DR, Fidias P, Lynch TJ, Rabindran SK, McGinnis JP, Wissner A, Sharma SV, Isselbacher KJ, Settleman J and Haber DA (2005) Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc Natl Acad Sci U S A* **102**:7665-7670.

Laursen LS and Oxvig C (2005) Real-time measurement in living cells of insulin-like growth factor activity using bioluminescence resonance energy transfer. *Biochem Pharmacol* **69**:1723-1732.

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**:2129-2139.

Minna JD and Dowell J (2005) Erlotinib hydrochloride. *Nat Rev Drug Discov* **Suppl**:S14-15.

Olive DM (2004) Quantitative methods for the analysis of protein phosphorylation in drug development. *Expert Rev Proteomics* **1**:327-341.

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**:1497-1500.

Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H (2004a) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* **101**:13306-13311.

Pao W and Miller VA (2005) Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* **23**:2556-2568.

Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H (2005) Acquired Resistance of Lung Adenocarcinomas to Gefitinib or Erlotinib Is Associated with a Second Mutation in the EGFR Kinase Domain. *PLoS Med* **2**:e73.

Pao W, Miller VA, Venkatraman E and Kris MG (2004b) Predicting sensitivity of non-small-cell lung cancer to gefitinib: is there a role for P-Akt? *J Natl Cancer Inst* **96**:1117-1119.

Perez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, Rigas J, Clark GM, Santabarbara P and Bonomi P (2004) Determinants of tumor response and survival with erlotinib in patients with non--small-cell lung cancer. *J Clin Oncol* **22**:3238-3247.

Perez-Soler R, Delord JP, Halpern A, Kelly K, Krueger J, Sureda BM, von Pawel J, Temel J, Siena S, Soulieres D, Saltz L and Leyden J (2005) HER1/EGFR inhibitor-associated rash: future directions for management and investigation outcomes from the HER1/EGFR inhibitor rash management forum. *Oncologist* **10**:345-356.

Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. *Cell* **103**:211-225.

Schlessinger J (2002) Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* **110**:669-672.

Schulze WX, Deng L and Mann M (2005) Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* **1**:2005 0008.

Sordella R, Bell DW, Haber DA and Settleman J (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* **305**:1163-1167.

Sorkin A, McClure M, Huang F and Carter R (2000) Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr Biol* **10**:1395-1398.

Spalding TA, Burstein ES, Brauner-Osborne H, Hill-Eubanks D and Brann MR (1995) Pharmacology of a constitutively active muscarinic receptor generated by random mutagenesis. *J Pharmacol Exp Ther* **275**:1274-1279.

Tateishi M, Ishida T, Mitsudomi T, Kaneko S and Sugimachi K (1990) Immunohistochemical evidence of autocrine growth factors in adenocarcinoma of the human lung. *Cancer Res* **50**:7077-7080.

Tyagi P (2005) Updated Data from the Iressa Survival in Lung Cancer Trial. *Clin Lung Cancer* **6**:340-342.

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Fig. 1. (A) Dose response curve for agonist EGF in EGFR/Grb2 BRET-2 assay. Wild type EGFR is co-expressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP-kinase signaling (see materials and methods). (B) Time course of EGF induced BRET-2 signal increase in the EGFR/Grb2 BRET-2 assay. Wild type EGFR is co-expressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor the increase of the EGFR BRET-2 signal over a time period of 20 minutes after addition of 17 nM EGF (see materials and methods) (C) Antagonism of EGF (0.33 nM) induced EGFR responses in EGFR BRET/Grb2 assays. Wild type EGFR is co-expressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP-kinase signaling (see materials and methods). Dose-responses for EGFR inhibitors Iressa (■), Tarceva (▲), AG1478 (▼) and PD168393 (◆) are compared.

Fig. 2. Constitutive and EGF-induced activities of mutant EGFR isoforms, and inhibition of their constitutive activities by small molecule EGFR inhibitor Iressa. Wild type EGFR-Luc or mutant EGFR-Luc isoforms are co-expressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP-kinase signaling (see materials and methods).(A) EGFR wildtype (WT) (B) EGFR G719C (C) EGFR L858R (D) EGFR Δ752-759 (E) EGFR Δ747-749 A750P. Dashed lines in figure 2A-E indicate EC_{50} for EGF responses and IC_{50} for Iressa responses at the wild type EGFR. Open symbols: EGF, filled symbols: Iressa; no ligand (no lig.)

Fig. 3. Differential affects of somatic EGFR mutations on constitutive EGFR signaling and EGF-induced signaling. The indicated wild type or mutant EGFR isoforms are co-expressed with GFP2-Grb2 (A), Stat5A-GFP2 (B), GFP2-p85 (C and E), or GFP2-PLCγ1 (E), in HEK293T cells and analyzed as described in Materials and Methods. The EGF-induced signal (0.33 nM EGF,) plus the constitutive activity for the wild type EGFR (filled bars) are normalized to 100%. The BRET-2 signal of the mutant

Molecular Pharmacology Fast Forward. Published on September 12, 2006 as DOI: 10.1124/mol.106.027656
This article has not been copyedited and formatted. The final version may differ from this version.

EGFR isoforms is expressed as a % of wild type EGFR responses and derived from ratios between the *Renilla* luciferase emission and the GFP2 emission corrected by the background emissions of non-transfected cells. Open bars: constitutive EGFR activity, filled bars: constitutive EGFR activity plus EGF induced activity. The following EGF stimulated wild type EGFR BRET-2 signals for the studied EGFR signaling pathways were observed: MAP-kinase pathway: 0.21 +/- 0.06 (-EGF), 0.55 +/- 0.02 (+ EGF); p85/PI3K pathway: 0.11 +/- 0.03 (- EGF), 0.21 +/- 0.03 (+EGF); STAT5 pathway: 0.08 +/- 0.008 (-EGF), 0.17 +/- 0.09 (+EGF); PLCγ1 pathway: 0.09 +/- 0.009 (-EGF), 0.14 +/- 0.03 (+ EGF).

Fig 4. Iressa and Tarceva are partial inhibitors of high constitutively active EGFR receptors that bear the resistant mutation T790M in combination with the L858R or Δ747-749 A750P mutations. Mutant EGFR isoforms are co-expressed with GFP2-p85 in HEK293T cells and analyzed in the EGFR/p85 BRET-2 assay, monitoring PI3K/Akt pathway signaling. Dose responses for the activation of EGFR WT and EGFR L858R T790M with EGF are shown in A. Dose responses for the inhibition of constitutive receptor activity with the small molecule inhibitor Iressa (▲) and Tarceva (●) are compared in B-C. (A) EGFR WT and EGFR L858R T790M (B) EGFR T790M (C) EGFR L858R T790M (D) EGFR Δ747-749 A750P.

Fig. 5. Constitutive activity of mutant EGFR isoforms leads to downstream ERK1/2 activation. Mutant EGFR isoforms are co-expressed with GFP2-Grb2 in HEK293T cells. Cells were either untreated (-) or treated (+) with 25 nM EGF for 5 minutes, lysed and analyzed by 12% SDS-PAGE and western blotting using antibodies recognizing human phosphorylated ERK1/2, ERK1/2 protein or *Renilla* luciferase. Lanes 1 and 2: no receptor control (GFP2-Grb2 alone), lanes 3 and 4: EGFR-Luc L858R T790M, lanes 5 and 6: EGFR-Luc L858R, lanes 7 and 8: wild type EGFR-Luc. Phospho-ERK1/2 antibody (Ab) (upper), control protein ERK1/2 Ab (middle), control *Renilla* luciferase Ab (lower).

Table 1. Pharmacology of Iressa and Tarceva on constitutive active EGFR isoforms**.**

The EGF EC_{50}, Iressa and Tarceva IC_{50} values for wild type and mutant EGFR isoforms were compared and tested for statistically significant differences by a 2-tailed unpaired t-test. Statistically significant differences are labeled as follows: $P<0.0001$ (*), $P=0.0002$ ($), $P=0.0006$ (#), $P=0.041$ (&), not determined (n.d.). The mean and standard error of the mean (SEM) for all values are shown.

	EC50 -Log [EGF] M	**n**	**IC50 - Log [Iressa] M**	**n**	**IC50 - Log [Tarceva] M**	**n**	**EGFR signaling pathway**
EGFR WT	10.14 +/- 0.01	32	6.59 +/- 0.08	15	6.83 +/- 0.08	15	
EGFR L858R	9.63 +/- 0.03*	8	7.59 +/- 0.05*	4	8.04 +/- 0.04*	4	**MAP kinase (Grb2)**
EGFR Δ752-759	9.71 +/- 0.06*	8	7.88 +/- 0.06*	4	8.23 +/- 0.05*	4	
EGFR Δ747-749 A750P	9.69 +/- 0.08*	7	7.64 +/- 0.03*	3	8.00 +/- 0.06*	4	
EGFR G719C	9.78 +/- 0.04*	8	7.41 +/- 0.10$^{\$}$	4	8.05 +/- 0.06*	4	
EGFR WT	9.97 +/- 0.09	6	n.d	3	n.d	3	
EGFR L858R	9.91 +/- 0.05	3	8.46 +/- 0.10	3	8.88 +/- 0.03	3	**STAT (STAT5A)**
EGFR Δ752-759	9.79 +/- 0.17	3	8.45 +/- 0.01	3	8.74 +/- 0.19	3	
EGFR WT	10.22 +/- 0.02	6	8.00 +/- 0.15	6	8.03 +/- 0.03	7	
EGFR L858R	9.36 +/- 0.21$^{\#}$	3	8.33 +/- 0.11	3	8.79 +/- 0.06*	3	**PI3K/Akt (p85)**
EGFR Δ752-759	9.25 +/- 0.18*	3	8.56 +/- 0.09$^{\&}$	3	8.60 +/- 0.09*	3	
EGFR WT	10.15 +/- 0.03	9	n.d.	4	n.d.	4	
EGFR L858R	9.89 +/- 0.02*	5	8.04 +/- 0.15	4	8.50 +/- 0.11	4	**PLCy1-calcium (PLCy1)**
EGFR Δ752-759	9.93 +/- 0.07	5	8.32 +/- 0.10	5	8.42 +/- 0.14	5	

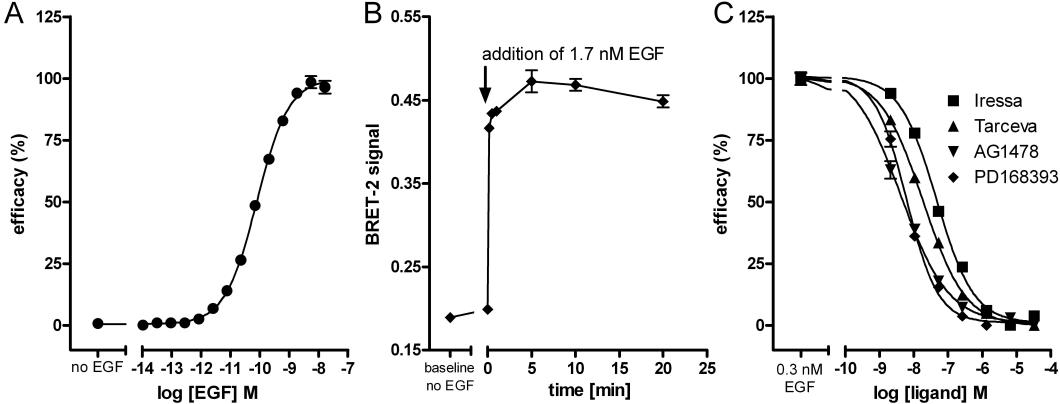
A
efficacy (%)
125
100
75
50
25
0
no EGF
-14 -13 -12 -11 -10 -9 -8 -7
log [EGF] M
B
BRET-2 signal
0.55
0.45
0.35
0.25
0.15
addition of 1.7 nM EGF
baseline
no EGF
0 5 10 15 20 25
time [min]
C
efficacy (%)
125
100
75
50
25
0
Iressa
Tarceva
AG1478
PD168393
0.3 nM
EGF
-10 -9 -8 -7 -6 -5 -4
log [ligand] M

Figure 1

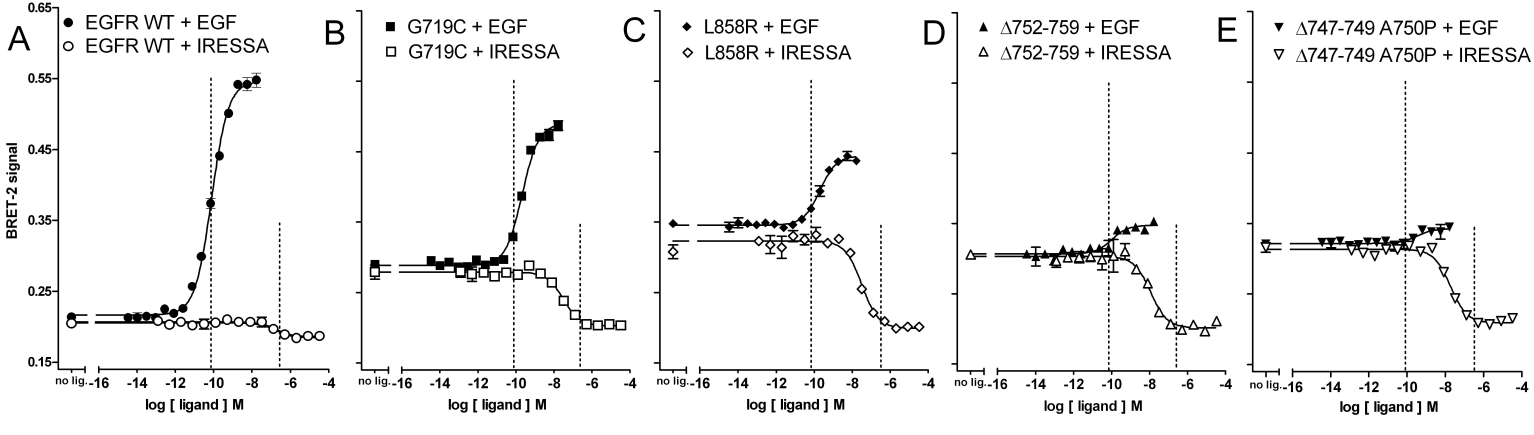
A
EGFR WT + EGF
EGFR WT + IRESSA
B
G719C + EGF
G719C + IRESSA
C
L858R + EGF
L858R + IRESSA
D
Δ752-759 + EGF
Δ752-759 + IRESSA
E
Δ747-749 A750P + EGF
Δ747-749 A750P + IRESSA
BRET-2 signal
0.65
0.55
0.45
0.35
0.25
0.15
no lig.
-16
-14
-12
-10
-8
-6
-4
log [ligand] M

Figure 2

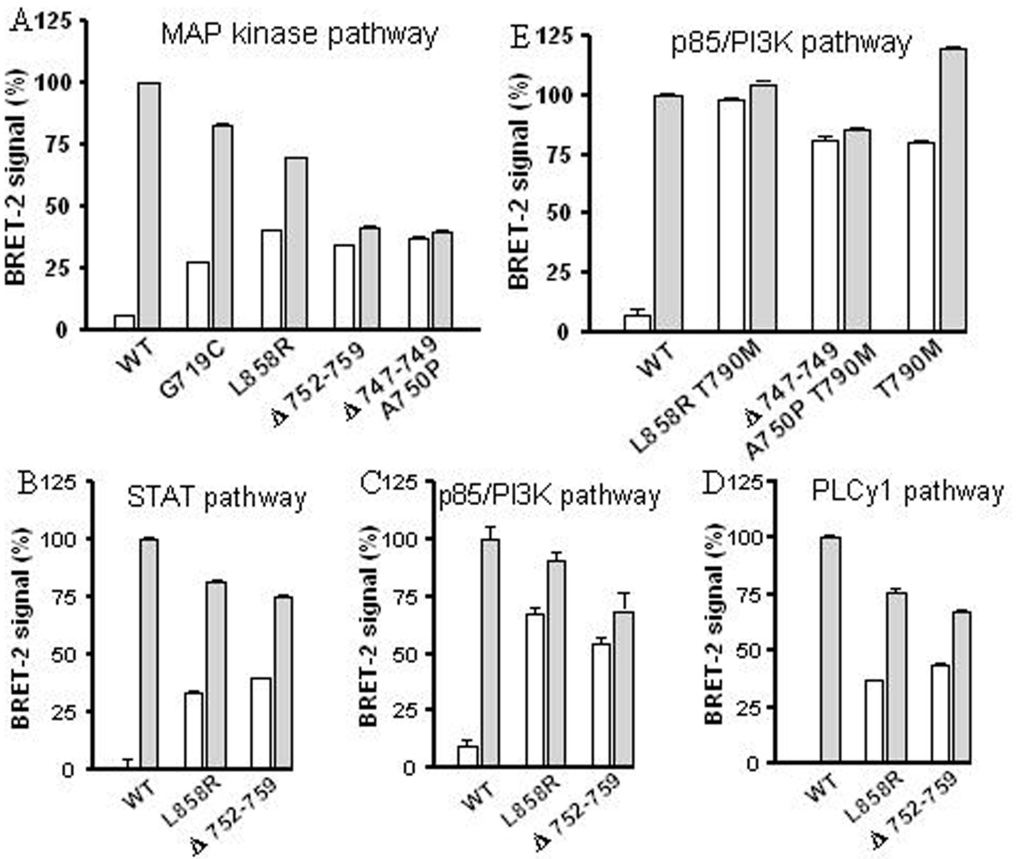
A
MAP kinase pathway
BRET-2 signal (%)
125
100
75
50
25
0
WT
G719C
L858R
Δ752-759
Δ747-749 A750P
E
p85/PI3K pathway
BRET-2 signal (%)
WT
L858R T790M
Δ747-749 A750P T790M
T790M
B
STAT pathway
BRET-2 signal (%)
WT
L858R
Δ752-759
C
p85/PI3K pathway
BRET-2 signal (%)
WT
L858R
Δ752-759
D
PLCγ1 pathway
BRET-2 signal (%)
WT
L858R
Δ752-759

Figure 3

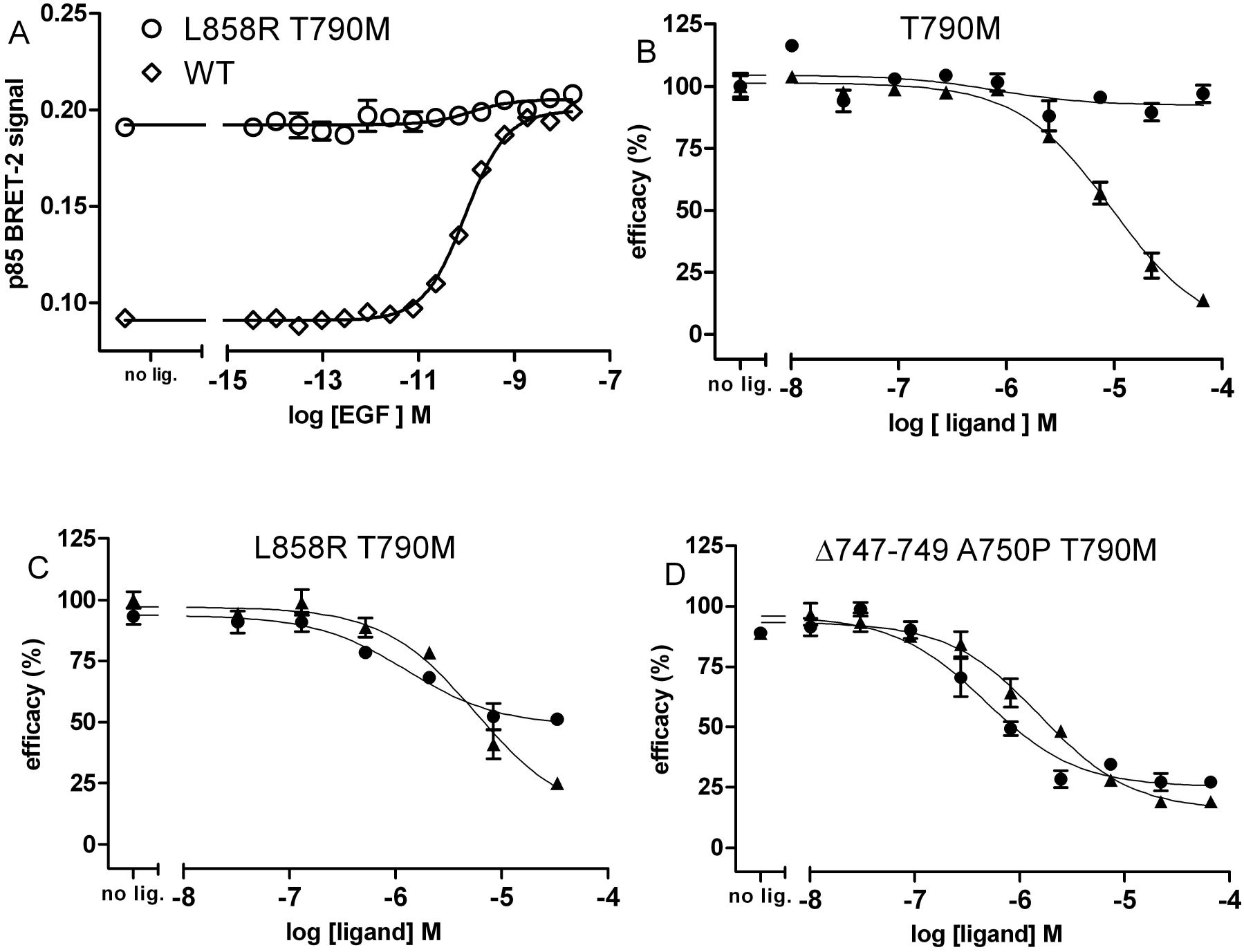
A
L858R T790M
WT
p85 BRET-2 signal
0.25
0.20
0.15
0.10
no lig.
-15
-13
-11
-9
-7
log [EGF] M
B
T790M
efficacy (%)
125
100
75
50
25
0
no lig.
-8
-7
-6
-5
-4
log [ligand] M
C
L858R T790M
efficacy (%)
125
100
75
50
25
0
no lig.
-8
-7
-6
-5
-4
log [ligand] M
D
Δ747-749 A750P T790M
efficacy (%)
125
100
75
50
25
0
no lig.
-8
-7
-6
-5
-4
log [ligand] M

Figure 4

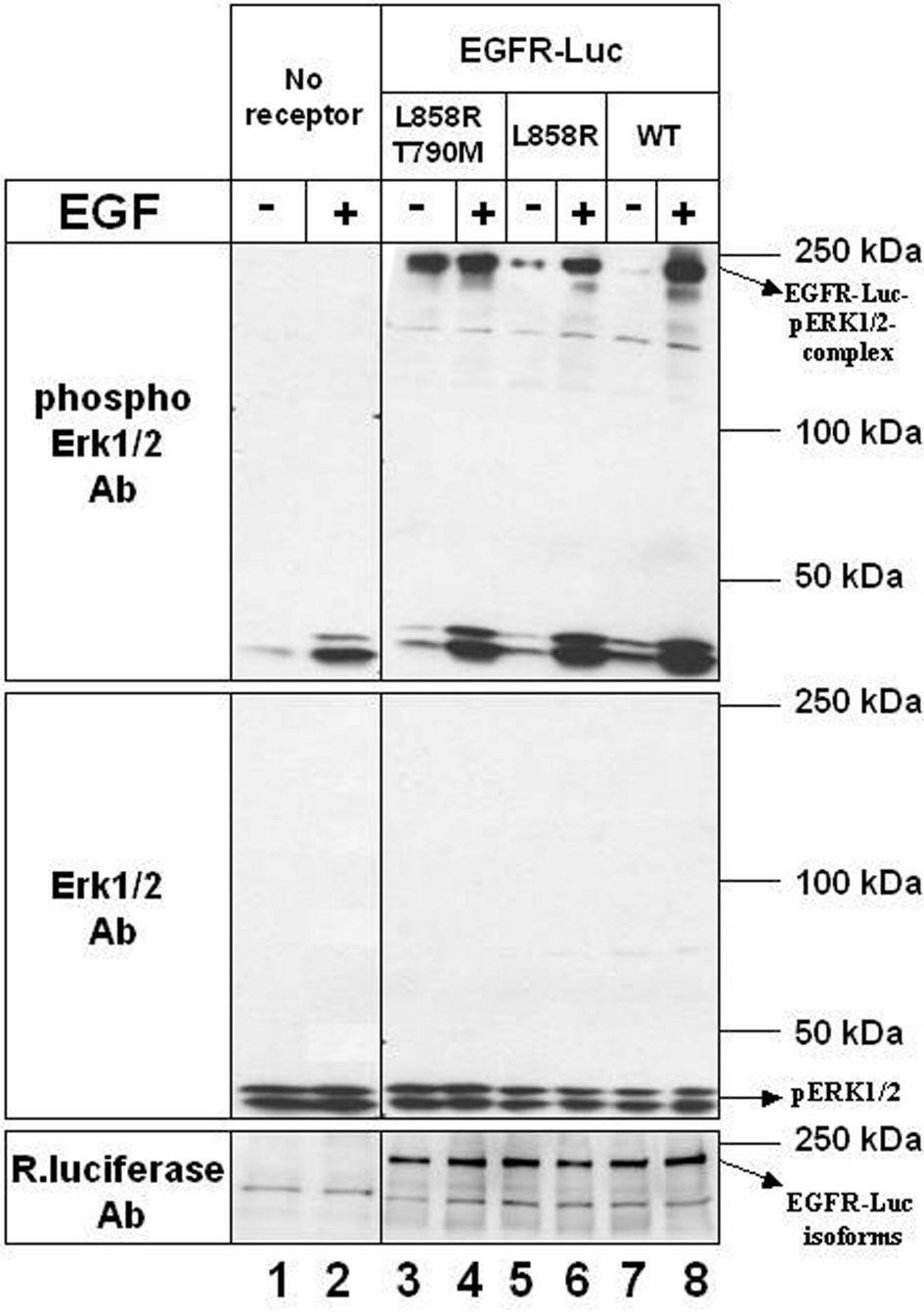
No receptor
EGFR-Luc
L858R T790M
L858R
WT
EGF
- + - + - + - +
250 kDa
EGFR-Luc-pERK1/2-complex
phospho Erk1/2 Ab
100 kDa
50 kDa
250 kDa
Erk1/2 Ab
100 kDa
50 kDa
pERK1/2
R.luciferase Ab
250 kDa
EGFR-Luc isoforms
1 2 3 4 5 6 7 8

Figure 5