### Table S1.

#### Kinetic Constants of EGFR Variants Panel with Gefitinib

<table>
<thead>
<tr>
<th></th>
<th>Calc. $t_{1/2}$ (min)</th>
<th>Rel. $t_{1/2}$</th>
<th>$V_{\text{release,Gef}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87:EGFR</td>
<td>14.45</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>U87:EGFRvIII</td>
<td>10.05</td>
<td>0.70</td>
<td>1.44</td>
</tr>
<tr>
<td>U87:EGFR L858R</td>
<td>20.90</td>
<td>1.45</td>
<td>0.69</td>
</tr>
<tr>
<td>U87:EGFRdel746-750</td>
<td>101.02</td>
<td>6.99</td>
<td>0.14</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY DATA

Figure S1. EGFR-alleles exhibit differential growth inhibition in response to erlotinib treatment. A, LN229MG cells were transduced with wild type-EGFR, EGFRvIII, EGFR L858R, or EGFRdel746-750. Cells were treated or untreated with EGF 30 min prior to harvest, as shown. Cells were then lysed and analyzed by immunoblot. Signaling downstream of EGFR was analyzed using antisera to total and phospho-specific proteins shown. B, The LN229MG panel was treated for 24 hours with three doses of erlotinib, as shown. Cells were analyzed for their DNA content, measured by propidium iodide staining. For each sample, the proportion of cells in S and G2/M phases was combined and graphed.
Figure S2. EGFR-alleles exhibit differential cell viability in response to erlotinib treatment. A, The U87MG panel was treated for three days with three doses of erlotinib, as shown. Cell viability was measured using the WST-1 reagent and scaled as a percent of the control (DMSO-treated). Treatments were completed in triplicate with error bars representing one standard deviation. B, The above experiment was repeated using the LN229MG panel.
**Figure S3.** EGFR fluorescent affinity probe ([16]) shows high specificity for common EGFR-alleles. The fluorescence affinity probe was previously shown to have high selectivity for analog-sensitive (as) alleles of EGFR. Here we show that a single band of staining at 170kDa (corresponding to EGFR) is seen upon [16] treatment of cells expressing EGFR, EGFR L858R, or EGFRdel746-750. Cells expressing EGFRvIII exhibit a band of staining at 145kDa, corresponding to this truncated form of the kinase. Therefore, the fluorescent affinity probe ([16]) has a high specificity for the EGFR wild-type kinase, as well as the other mutant EGFR-alleles analyzed.
Figure S4. Erlotinib treatment achieves different levels of kinase site occupancy in EGFR-alleles. The LN229MG panel was treated overnight with varying doses of erlotinib and stimulated for 30 minutes with 100ng/mL EGF. Cells were then subjected to a 25 minute pulse-chase with 60µM [16]. Cells were then lysed and separated by SDS-PAGE. Gels were scanned on a Typhoon fluorescence imager using a 488nm excitation laser. The level of fluorescence corresponds to the amount of kinase active site that is unbound by erlotinib (100-% kinase site occupancy), and thus is available for probe binding. The fluorescence intensity for each treatment was quantified by densitometry and scaled as a percent of the EGF-stimulated control lane. At all erlotinib concentrations, the measured kinase site occupancy differed between EGFR-alleles. Results were completed in triplicate with error bars representing one standard deviation (*p<0.05, ***p<0.005).
**Figure S5.** Kinase site occupancy is a better biomarker than abundance of p-EGFR, for efficacy of erlotinib. **A,** For each *EGFR*-allele and at each dose of erlotinib, $100-\text{(% kinase site occupancy)}$ was assigned the x-coordinate and the relative number of cells in S and G2/M phases, the y-coordinate. This allowed for the quantification of the relationship between kinase site occupancy and drug efficacy for each EGFR-allele, as measured by the square of the correlation coefficient ($R^2$). The similar trajectory of the trend line for each EGFR-allele reveals that kinase site occupancy acts as a direct measure of cell cycle response; the different alleles will respond with similar cell cycle arrest if the same level of kinase site occupancy is reached. **B,** Similar results were seen in the LN229MG panel.
**Figure S6.** Kinase site occupancy does not correlate with p-EGFR. **A,** For each EGFR-allele and at each dose of erlotinib, 100-(% kinase site occupancy) was assigned the x-coordinate and the percent p-EGFR (Y1173) the y-coordinate. This allowed for the quantification of the relationship between kinase site occupancy and pelf levels. A correlation of $R=0.709$ was observed. **B,** The same analysis was applied to quantify the relationship between percent open kinase site and p-Tyr (4G10) levels. A correlation of $R=0.500$ was observed.
Figure S7. Kinase site occupancy is a better biomarker than abundance of p-EGFR, for efficacy of erlotinib. A, For each EGFR-allele and at each dose of erlotinib, the level of p-EGFR (Y1173), as determined by densitometry of LI-Cor analyzed western blots, was assigned the x-coordinate and the relative number of cells in S and G2/M phases, the y-coordinate. This allowed for the quantification of the relationship between p-EGFR (Y1173) levels and drug efficacy. We found a weak (R=0.710) correlation between p-EGFR (Y1173) levels and efficacy for the U87 panel. B, The same analysis was applied to the LN229 panel, where a correlation of R=0.522 was found between p-EGFR (Y1173) and efficacy.
**Figure S8.** Kinase site occupancy is a better biomarker than abundance of p-EGFR, for efficacy of erlotinib. For each EGFR-allele and at each dose of erlotinib, the global phosphorylation of EGFR (as measured by densitometry of p-Tyr (4G10) antibody staining from LI-Cor analyzed western blots) was assigned the x-coordinate and the relative number of cells in S and G2/M phases, the y-coordinate. This allowed for the quantification of the relationship between global phosphorylation of EGFR and drug efficacy. A weak correlation (R=0.523) was found in this analysis.
**Figure S9.** Kinase site occupancy is a better biomarker than abundance of p-EGFR, for efficacy of erlotinib. **A,** For each EGFR-allele and at each dose of erlotinib, the phosphorylation of EGFR at Y1173 (as measured by densitometry of p-EGFR (Y1173) antibody staining from LI-Cor analyzed western blots) was assigned the x-coordinate and the relative number of cells in S and G2/M phases, the y-coordinate. This allowed for the quantification of the relationship between p-EGFR (Y1173) levels and drug efficacy for each allele of EGFR, as measured by the square of the correlation coefficient ($R^2$). The different trajectory of the trend line for each EGFR-allele reveals that the different alleles will respond with different cell cycle arrest in response to the same decrease in p-EGFR. This suggests p-EGFR as a poor marker for cell cycle inhibition across mutant EGFR-alleles. **B,** The same analysis was applied to global phosphorylation of EGFR (as measured by densitometry of p-Tyr (4G10) staining from LI-Cor analyzed western blots).
**Figure S10.** Antiproliferative effects of erlotinib correlate poorly with abundance of p-EGFR. The LN229MG panel was treated with three doses of erlotinib, as shown, then pulsed with 100ng/mL EGF prior to harvesting. Phospho- and total protein levels were visualized by western blotting. While low dosages of erlotinib efficiently block p-EGFR (Y1173) in all cell lines, levels of p-ERK 1/2 (T202/Y204) and p-AKT (S473) were decreased in cells expressing NSCLC-derived EGFR L858R and EGFRdel746-750, compared with glioma-derived EGFRvIII, paralleling the antiproliferative response in Supplementary Figure 1b. Levels of kinase site occupancy more closely follow abundance of phosphorylated downstream molecules.
Figure S11. Immunoblot analysis of U87:EGFR T790M and LN229:EGFR T790M after treatment with erlotinib. A, Relevant doses of erlotinib showed no effect on abundance of p-AKT and p-ERK 1/2 in U87 cells expressing lung-derived EGFR T790M, which has a bulky gatekeeper residue and is resistant to EGFR TKI therapy. Since any effect on downstream signaling in these cells would reflect off-target (non-EGFR-mediated) effects of erlotinib, these data suggest that the changes in the abundance of p-AKT and p-ERK 1/2 seen in Figure 4 and Supplementary Figure 10 are due to the effects of erlotinib on each EGFR-allele. B, Similar results were observed in LN229 cells expressing lung-derived EGFR T790M.
Figure S12. Excel Analysis of Kinetics Data. Equation (2)

\[
EGFR: Erl + [16] \xrightleftharpoons[k_B]{k_U} EGFR + Erl + [16] \xrightarrow{k_2} EGFR: [16] + Erl
\]

(2)

illustrates the reaction between EGFR, erlotinib, and [16], with \( k_B \) representing the rate constant of erlotinib binding by EGFR, \( k_U \), the rate constant of erlotinib unbinding by EGFR, and \( k_2 \), the rate constant of [16] binding by EGFR. A, Corrected open kinase site values for the kinetic analysis of the interaction of erlotinib and the EGFR-allele panel (as determined in Figure 5) were best fit to an equation of the form of equation (3) for analysis.

\[
f(t) = \frac{At}{B + Ct + D}
\]

(3)

Values for all constants were determined using the Solver function of Microsoft excel to minimize the sum of the difference of the experimentally determined values for open kinase site and the value as determined by the best-fit equation. B-E, The best fit equations for U87:EGFR (B), U87:EGFRvIII (C), U87:EGFR L858R (D), and U87:EGFRdel746-750 (E), labeled as ‘Calc. data’ were graphed on the same axis as the experimental data, labeled as ‘Exp. data’ to check for a close fit. Best-fit equations tended to deviate from the experimental data at the 4-hour time point, but since these equations were used for \( t_{1/2} \) calculations, this deviation was acceptable.
**Figure S13.** Kinetics of gefitinib binding/unbinding differ across *EGFR*-alleles. **A** and **B,** The U87 panel was treated with 1µM gefitinib (**A**) or DMSO (**B**) for 24 hours and then pulsed with 60µM [16] for 1 minute, 10 minutes, 25 minutes, 1 hour, or 4 hours. In the drug treated experiment (**A**), the control was untreated and pulsed with [16] for 4 hours. As gefitinib cycles out of *EGFR,* the active site is irreversibly bound by [16], preventing its re-binding. Over time, this occurs with all *EGFR*-bound gefitinib. The rate with which this replacement occurs is related to the speed with which gefitinib is unbound by *EGFR.* The control (**B**) established the rate at which [16] alone binds each *EGFR*-allele (*k*₂ in Equation (2)). **C,** Analysis of (**A**) and (**B**) by densitometry allowed for the quantification of [16] binding over time in the presence or absence of erlotinib. The level of [16] staining of a single *EGFR*-allele (as a % of the 4hr control lane) determined in (**A**) was divided by the level of [16] staining of that same *EGFR*-allele (as a % of the 4hr control lane) determined in (**B**), allowing for the tracking of kinase site occupancy for each *EGFR*-allele over time. These data determine that gefitinib replacement occurs more quickly in glioma-derived EGFRvIII than in NSCLC-derived EGFR-mutants.
Table S1. Relative t_{1/2} values for panel of EGFR-alleles. Data from Supplementary Figure 13 was fit to an equation of the form of equation (1). The derived function was used to calculate the t_{1/2}, the time after which one half of the EGFR active site has been bound by [16]. The values of t_{1/2} represent the relative speed with which each allele of EGFR releases gefitinib. The relative t_{1/2} was calculated by scaling all values relative to the t_{1/2} of the wild-type kinase. The inverse of this value provides the rate, relative to EGFR^{WT}, with which the mutant EGFR-alleles release gefitinib, V_{release,Gef}, and is related to gefitinib’s period of occupancy of each kinase site.