Inhibiting Epidermal Growth Factor Receptor at a Distance

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S *Supporting Information*

ABSTRACT: The epidermal growth factor receptor (EGFR) tyrosine kinase is implicated in a large number of human cancers. Most EGFR inhibitors target the extracellular, growth factor-binding domain or the intracellular, ATP-binding domain. Here we describe molecules that inhibit the kinase activity of EGFR in a new way, by competing with formation of an essential intradimer coiled coil containing the juxtamembrane segment from each member of the receptor partnership. The most potent molecules we describe bind EGFR directly, decrease the proliferation of wild-type and mutant EGFR-dependent cells lines, inhibit phosphorylation of EGFR and downstream targets, and block coiled coil formation as judged by bipartite tetracysteine display. Potency is directly correlated with the ability to block coiled coil formation within full-length EGFR in cells.

The epidermal growth factor receptor (EGFR)[1−3] tyrosine kinase is implicated in a large number of human cancers.[4] Four EGFR inhibitors have been approved for use: cetuximab[5,6] is a monoclonal antibody that directly inhibits the binding of growth factors to the EGFR extracellular domain,[7] whereas gefitinib, erlotinib, and afatinib[8−11] are tyrosine kinase inhibitors (TKIs) that directly inhibit the binding of ATP to the intracellular catalytic domain.[4,12] Other molecules in these two categories, including reversible and irreversible TKIs that inhibit the drug-resistant EGFR double mutant, are in clinical development.[13−19] Here we describe molecules that inhibit EGFR in a third way, via allostery,[20,21] by blocking the formation of a coiled coil dimer in the juxtamembrane (JM) segment (Figure 1A) that is essential for assembly of the active, asymmetric kinase dimer.

Recently we reported, using a tool known as bipartite tetracysteine display,[22,23] that the binding of the epidermal growth factor (EGF)[24] to the extracellular domain of full-length EGFR[1−3] leads to the assembly of an antiparallel coiled coil composed of the JM segment from each member of the protein pair. The JM segment is located between the transmembrane helix and the kinase domain (Figure 1A) and is essential for kinase function.[25−27] EGFR variants that lack a JM segment[28] or contain amino acid substitutions that reduce α-helix propensity[26,29] are catalytically inactive. Other variants that disfavor assembly of the active, asymmetric kinase dimer[26] do not support formation of the JM coiled coil.[25] These observations suggest that ligands capable of inhibiting coiled coil formation should inhibit the EGFR kinase via an allosteric mechanism. Indeed, a polypeptide containing the EGFR JM
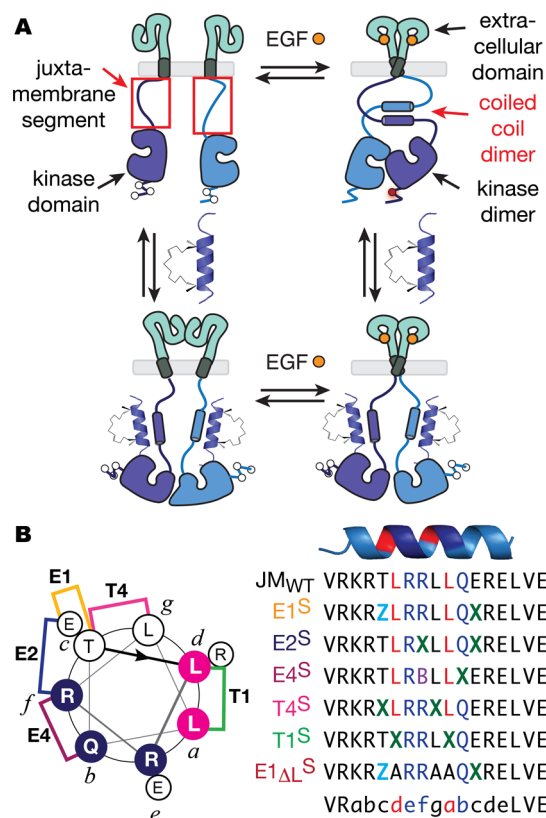
Figure 1. (A) Potential equilibria between EGFR monomers and dimers ± growth factor (EGF) and allosteric inhibitors. (B) Helical wheel representation and sequences of hydrocarbon-stapled peptides. Z, X, and B represent (R)-2-(7′-octenyl)alanine, (S)-2-(4′-pentenyl)-alanine, and (R)-2-(4′-pentenyl)alanine, respectively. Peptides constrained with a hydrocarbon staple are indicated with the superscript S.

segment fused to a polycationic region from HIV Tat (TE-64562) inhibits EGFR signaling, but neither its binding mode nor its mechanism of action is understood, as kinase activity itself was unaffected.[30]

Previous work has shown that peptides containing judiciously positioned i+3, i+4, and i+7 macrocyclic bridges (often referred to as hydrocarbon staples) can display improved α-helix content, protease resistance, and, in some cases, cellular uptake when compared to unmodified peptides with similar sequences.[31−33] These features make hydrocarbon-stapled peptides uniquely suited to evaluate the JM coiled coil as an

allosteric regulatory site for EGFR. To begin this evaluation, we synthesized five peptides comprising the 17-residue JM-A segment (EGFR residues 645−662) and a single hydrocarbon staple at one of five positions around the helix circumference (Figure 1B and Supporting Information, Figures S1 and S2). Four of the peptides (E1[S], E2[S], E4[S], and T4[S]) contain a hydrocarbon staple on the helix face opposite that used for EGF-stimulated coiled coil formation.[25] One peptide (T1[S]), prepared as a control, contains a hydrocarbon staple that blocks the helix face used for EGF-stimulated coiled coil formation.[25,26] Two additional peptides contain the unmodified JM-A sequence fused to a polycationic region of HIV Tat (TE-64562)[30] or not (JM-WT). As expected, all hydrocarbon-stapled peptides displayed greater α-helical content than JM-WT or TE-64562 (Figure S3). We reasoned that if the JM coiled coil regulates EGFR activity via allostery, then ligands E1[S], E2[S], E4[S], and T4[S] should inhibit EGFR activity and decrease the viability of EGFR-dependent cell lines, albeit to varying degree, whereas T1[S] and JM-WT should have little or no effect.

We evaluated the effect of each molecule on the viability of four EGFR-dependent cell lines that differ in cancer/tissue type as well as EGFR expression level and mutational state and one cell line that does not express EGFR (Figures 2 and S4 and
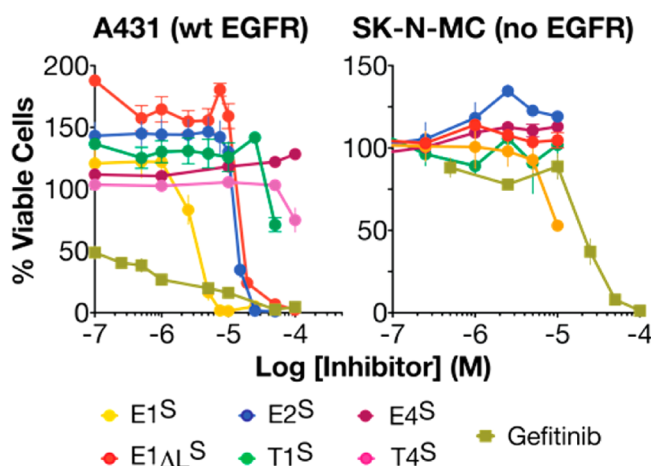
Figure 2. Effect of native and hydrocarbon-stapled peptides on cell proliferation. Plot of % viable cells remaining after 18 h treatment with [ligand] shown. Viability was assessed by monitoring oxyluciferin production by Ultra-Glow luciferase, a reaction that requires ATP. Error bars show standard error of the mean.

Table S3). A431 and H2030 cells express wild-type EGFR, whereas H3255 and H1975 cells express single (L858R) or double (L858R/T790M) mutant forms, respectively; SK-N-MC cells express ErbB2- 4 but not EGFR.[3,34−36] Examination of the dose−response curves reveals several trends. First, as expected, cells expressing wild-type EGFR (A431) or the L858R mutant (H3255) are sensitive to gefitinib in the expected concentration range, whereas those expressing the EGFR double mutant (H1975) or no EGFR (SK-N-MC) are not.[37] Second, none of the cells are sensitive to JM-WT, an unmodified (and non-cell-permeable) peptide containing the EGFR JM-A sequence; fusion of JM-WT to a polycationic region of HIV Tat results in moderate decreases in viability after 18 h incubation, as reported;[30] potency is mitigated significantly after 72 h, perhaps because of degradation (Figure S5).

Most importantly, all EGFR-expressing cell lines are sensitive to one or more hydrocarbon-stapled peptides, with potency following the order E1[S] > E2[S] ≫ T4[S] ≥ E4[S]. T1[S] was inactive in all cell lines tested. In all cases, the most potent inhibitor (E1[S]) carries the hydrocarbon bridge on the helix face that lies *opposite* that used for EGF-induced coiled coil formation,[25] whereas the least potent molecule (T1[S]) is bridged within this face, with the bridge replacing two leucine side chains that contribute to the antiparallel coiled coil interface.[26] Both of these molecules gain entry to the cytosol, as judged by a previously reported[38,39] image-based translocation assay (Figure S11). E1[S] is 10-fold more potent than E1$_{\Delta L}$[S], in which the two leucines are replaced by alanine, and was between 2 and 10 times more potent than the previously reported TE-64562 peptide,[30] with the largest difference in H3255 cells that express L858R EGFR. These observations suggest that the decrease in cell viability observed in the presence of E1[S] results from a direct interaction of the helical peptide mimetic with the JM region of EGFR.

Activation of EGFR upon growth factor binding leads to a well-characterized pattern of Tyr and Ser/Thr autophosphorylation events that initiate downstream signaling networks.[40] Molecules that block growth factor binding to the extracellular domain, or ATP binding to the intracellular kinase domain, inhibit the phosphorylation of both EGFR and downstream factors such as Erk and Akt.[4] We used immunoblots to evaluate whether the effects of native and hydrocarbon-stapled peptides on the viability of EGFR-dependent cell lines correlated with their effects on EGFR phosphorylation and the phosphorylation of downstream factors. We probed specifically for phosphorylation at EGFR tyrosines 845, 1045, 1068, 1086, 1148, and 1173 and for phospho-Akt and phospho-Erk1/2 (Figures 3 and S6).

Incubation of A431 cells with 1−50 μM E1[S] led to a dose-dependent decrease in EGFR phosphorylation at positions Y845, Y1045, Y1086, and Y1173 (Figure 3, red bars); phosphorylation at Y1068 and Y1148 was affected minimally, if at all (see also Figure S7). A431 cells treated with E1[S] also showed decreased levels of phospho-Akt and phospho-Erk; the levels of EGFR, Akt, and Erk themselves were unaffected. The pattern of phosphorylation changes induced by E1[S] paralleled those observed with TE-64562. E2[S] and E1$_{\Delta L}$[S], which had more modest effects on cell viability (Figure 2), caused little or no decrease in phosphorylation at any position, whereas T1[S], E4[S], and T4[S] led to small increases in phosphorylation at many positions. Thus, in A431 cells, there is a correlation between the effect of hydrocarbon-stapled peptides on cell viability and decreases in EGFR autophosphorylation and downstream signaling.

Two additional experiments were performed to evaluate whether the E1[S]-induced viability changes and decreases in EGFR and Erk/Akt phosphorylation resulted from a direct interaction with the EGFR JM segment. First, we evaluated the extent to which biotinylated analogues of E1[S] and T1[S] as well as JM-WT and E1$_{\Delta L}$[S] ([B]E1[S], [B]T1[S], [B]JM-WT, and [B]E1$_{\Delta L}$[S], respectively) could sequester full-length EGFR (wtEGFR) from transiently transfected CHO-K1 cell lysates. Each biotinylated analogue (25 μM) was incubated for 1 h with lysates from wtEGFR-expressing cells, and then with Mag-Sepharose streptavidin beads overnight. A mock reaction lacking a biotinylated analogue was run alongside. After washing, the sequestered proteins were eluted, resolved by SDS-PAGE, probed with a commercial anti-EGFR antibody,
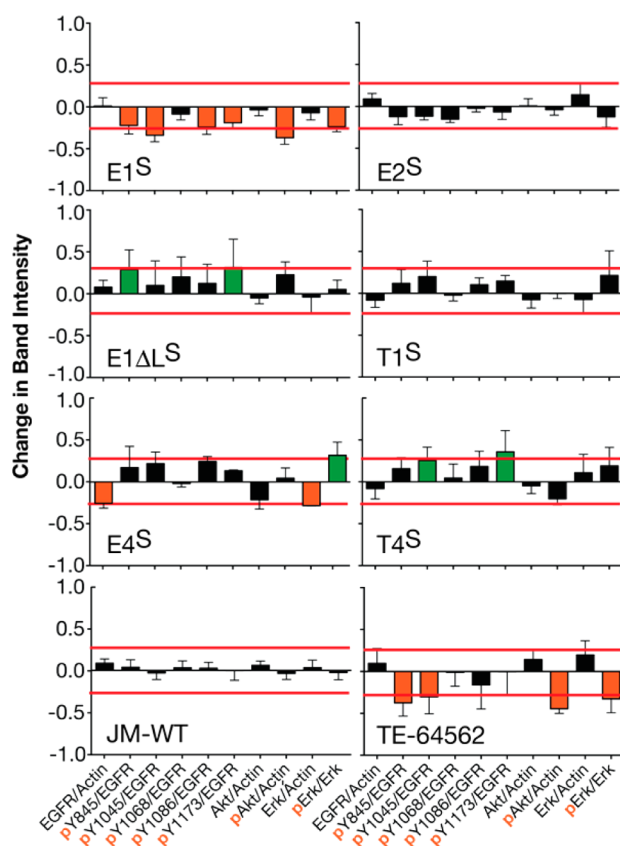
Figure 3. Effect of native and hydrocarbon-stapled peptides on phosphorylation of EGFR, Akt, and Erk1/2. A431 cells were treated with 10 μM of the ligand shown for 2 h, stimulated with 10 ng/mL EGF, and then lysed, immunoblotted, and visualized. Plots show the increase (green) or decrease (red) in intensity of the indicated phospho-protein band between treated and untreated cells. Error bars represent the standard error of the mean over at least four trials.

visualized with a horseradish peroxidase-tagged mouse anti-rabbit secondary antibody, and quantified with chemiluminescent detection. $^{B}E1^{S}$ and, less effectively, $^{B}E1_{\Delta L}^{S}$ sequestered full-length, wild-type EGFR from the cell lysates, whereas $^{B}T1^{S}$ and ^{B}JM-WT did not (Figure 4). Little or no EGFR was sequestered when no biotinylated peptide was added (mock), providing additional support for a direct interaction between the hydrocarbon-stapled peptide $E1^{S}$ and the JM segment of wild-type EGFR.

Finally, we made use of a previously reported bipartite tetracysteine display[22,23] assay to probe whether $E1^{S}$ inhibited
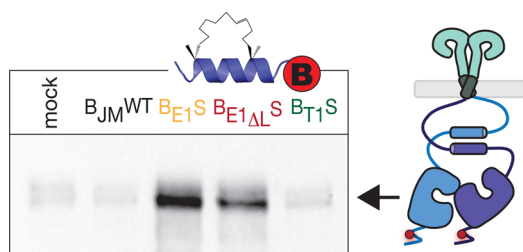
Figure 4. $^{B}E1^{S}$ sequesters wtEGFR from CHO-K1 cell lysates. Lysates were treated with 25 μM of the biotinylated peptide shown (1 h) and then incubated with streptavidin-coated beads overnight. Sequestered proteins were eluted, electrophoresed, and immunoblotted to detect EGFR. Band intensities were measured using ImageJ.[41]

intradimer coiled coil formation within the JM region of full-length EGFR on the mammalian cell surface (Figure 5).[25] We
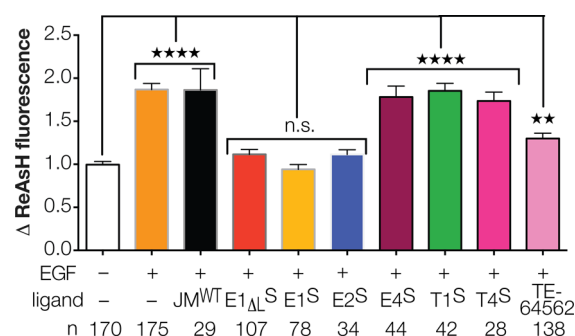
Figure 5. Monitoring EGFR coiled coil formation using TIRF-M and bipartite tetracysteine display. CHO cells were transfected with EGFR CC$_{H}$-1, treated with 1 μM ligand for 1 h, stimulated with 100 ng/mL EGF for 30 min, and labeled with ReAsH.[25] Plot shows the change in ReAsH fluorescence of n cells after correction for differences in expression. Errors represent standard error of the mean: $**p < 0.01$, $****p < 0.0001$; one-way ANOVA with Bonferroni post-analysis accounting for multiple comparisons.

used CHO cells expressing an EGFR variant (CC$_{H}$-1) with a cysteine pair within the JM whose location supports ReAsH binding and fluorescence upon EGF-induced coiled coil assembly.[25] We reasoned that if $E1^{S}$ inhibits formation of the JM coiled coil, it should also decrease the ability of CC$_{H}$-1 to bind ReAsH and fluoresce in the presence of EGF.

CHO cells transiently expressing the EGFR variant CC$_{H}$-1 on the cell surface were exposed to native and hydrocarbon-stapled peptides, stimulated with EGF, and incubated with ReAsH, and the fluorescence increase due to ReAsH was quantified using total internal reflectance fluorescence microscopy (TIRF-M). Treatment with EGF alone led to the expected increase in ReAsH fluorescence at the cell surface; this increase was unchanged by the presence of JM-WT, $E4^{S}$, $T1^{S}$, or $T4^{S}$, consistent with their inability to decrease the viability of EGFR-expressing cells (Figure 2) and sequester transfected EGFR from CHO cell lysates (Figure 3). However, treatment of cells with 1 μM $E1^{S}$, $E2^{S}$, $E1_{\Delta L}^{S}$, and, to a lesser extent, TE-64562 led to a significant loss in ReAsH fluorescence, which we infer to represent a loss in coiled coil structure. At a lower concentration only $E1^{S}$ and $E1_{\Delta L}^{S}$ reduced the ReAsH signal (Figure S8). Identical results were observed when cells were treated first with EGF and then with peptide (Figure S9). No peptide tested affected ReAsH fluorescence in the absence of EGF (Figure S10). These data support a model in which $E1^{S}$, $E1_{\Delta L}^{S}$, $E2^{S}$, and, to a lesser extent, TE-64562 interact with the EGFR JM segment to inhibit formation of the intradimer coiled coil. Taken with the cell viability, pull-down, and immunoblotting experiments, we propose that $E1^{S}$ allosterically inhibits EGFR by disrupting intradimer coiled coil formation within the juxtamembrane segment.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Descriptions of peptide synthesis and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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