Inhibiting Epidermal Growth Factor Receptor at a Distance

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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 cell 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: cetuximab5,6 is a monoclonal antibody that directly inhibits the binding of growth factors to the EGFR extracellular domain,7 whereas gefitinib, erlotinib, and afatinib8−11 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 EGFR1−3 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 segment28 or contain amino acid substitutions that reduce α-helix propensity26,29 are catalytically inactive. Other variants that disfavor assembly of the active, asymmetric kinase dimer26 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 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 (E1S, E2S, E4S, and T4S) contain a hydrocarbon staple on the helix face opposite that used for EGF-stimulated coiled coil formation. One peptide (T1S), prepared as a control, contains a hydrocarbon staple that blocks the helix face used for EGF-stimulated coiled coil formation. Two additional peptides contain the unmodified JM-A sequence fused to a polycationic region of HIV Tat (TE-64562) 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 E1S, E2S, E4S, and T4S should inhibit EGFR activity and decrease the viability of EGFR-dependent cell lines, albeit to varying degree, whereas T1S 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 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. 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; the levels of EGFR, Akt, and Erk themselves were unaffected. The pattern of phosphorylation changes induced by E1S–paralleled those observed with TE-64562. E2S and E1LΔS, which had more modest effects on cell viability (Figure 2), caused little or no decrease in phosphorylation at any position, whereas T1S, E4S, and T4S 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 E1S-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 E1S and T1S as well as JM-WT and E1LΔS 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, and visualized by autoradiography. The data showed that E1S and T1S bound to EGFR with higher affinity than T1S and JM-WT, and E1LΔS, respectively. The data suggest that the hydrocarbon stapled peptide E1S binds to the JM-A sequence on EGFR and prevents binding of EGF, resulting in decreased EGFR phosphorylation at downstream signaling molecules such as Erk and Akt. These findings support the hypothesis that E1S inhibits EGFR activity through allosteric regulation.
visualized with a horseradish peroxidase-tagged mouse anti-rabbit secondary antibody, and quantified with chemiluminescent detection. BE1S and, less effectively, BE1ΔLS sequestered full-length, wild-type EGFR from the cell lysates, whereas BT1S and BJM-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 E1S and the JM segment of wild-type EGFR.

Finally, we made use of a previously reported bipartite tetracysteine display22,23 assay to probe whether E1S inhibited intradimer coiled coil formation within the JM region of full-length EGFR on the mammalian cell surface (Figure 5).25 We used CHO cells expressing an EGFR variant (CC1H-CR) 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 E1S inhibits formation of the JM-coiled coil, it should also decrease the ability of CC1H-CR to bind ReAsH and fluoresce in the presence of EGF.

CHO cells transiently expressing the EGFR variant CC1H-CR 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, E4S, T1S, or T4S, 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 E1S, E2S, E1ΔLS, 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 E1S and E1ΔLS 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 E1S, E1ΔLS, E2S, 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 E1S allosterically inhibits EGFR by disrupting intradimer coiled coil formation within the juxtamembrane segment.
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