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Influence of Macrocyclization on Allosteric, Juxtamembrane-Derived, Stapled Peptide Inhibitors of the Epidermal Growth Factor Receptor (EGFR)

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ABSRACT: The hydrocarbon-stapled peptide E1S allosterically inhibits the kinase activity of the epidermal growth factor receptor (EGFR) by blocking a distant but essential protein–protein interaction: a coiled coil formed from the juxtamembrane segment (JM) of each member of the dimeric partnership. Macrocyclization is not required for activity: the analogous unstapled (but alkene-bearing) peptide is equipotent in cell viability, immunoblot, and bipartite display experiments to detect coiled coil formation on the cell surface.

Recently we reported1 a group of hydrocarbon-stapled peptides2 that allosterically inhibit the kinase activity of the epidermal growth factor receptor (EGFR). The molecules we described block a protein–protein interaction distal to the kinase domain that is nonetheless essential for kinase function.2–4 Specifically, these molecules block assembly of an antiparallel coiled coil containing the juxtamembrane (JM) segment from each member of the dimeric receptor partnership (Figure 1A). Formation of the antiparallel JM coiled coil is conformationally coupled to assembly of the catalytically active asymmetric kinase dimer.2–4 The most potent molecule we described, E1S, contains the sequence from the EGFR JM-A region (residues 650 to 666), constrained by an i to i + 7 macrocyclic cross-link between residues 5 and 12 (654 and 661 according to EGFR numbering) (Figure 1B). In E1S, the cross-link lies at position “c” of the heptad repeat, on the helix face opposite the “a” and “d” positions used for coiled coil formation within intact EGFR dimers.4 E1S decreases the viability of EGFR-dependent cell lines, inhibits EGFR autophosphorylation, and blocks coiled coil formation in live cells.1 Here we report that macrocyclization per se is not required for any of these metrics: the analogous unstapled (but alkene-bearing) peptides are equipotent in cell viability, immunoblot, and bipartite tetracysteine display experiments that monitor coiled coil formation within the JM on the mammalian cell surface.

In our previous work we noticed that the inhibitory potency of a JM-derived stapled peptide in cell-based proliferation assays was highly dependent on the location and identity of the macrocyclic cross-link. Although at least three molecules prepared previously (E1S, E2S, T4S) contained a cross-link that should permit formation of a coiled coil dimer with a single EGFR JM segment, only one (E1S) was highly active.1 We prepared a series of E1S variants to investigate this structure–activity relationship further (Figure 2A and Figure S1−2). One variant (JMAlb) contained a pair of α-helix-promoting α-aminoisobutyric acid (Aib) residues at positions 5 and 12, replacing the alkene-bearing residues required for macrocyclization of E1S. JMAlb thereby decouples the functional contribution of α-carbon quaternization and macrocyclization. A second, “unstapled” variant (E1U) contained the alkene-bearing residues required for macrocyclization of E1S, but no macrocyclization reaction was performed. Analogous “unstapled” versions of the remaining stapled peptides reported previously1 (E2U, E4U, T1U, and T4U) were also prepared (Figure 1B), as were three new, stapled peptides (E2.2S, T4.2S, and E2.3S, Figure 1C) designed to further probe the role of staple placement on EGFR inhibition. Two new molecules, E2.2S and T4.2S, contain a single i to i + 3 cross-link that is displaced by one helix turn from its position in E2U and T4U, respectively; the last, E2.3S, contains an i to i + 7 cross-link (like E1S) between residues located at two j positions of the heptad repeat.

As expected,1 when examined using circular dichroism (CD) spectroscopy all unstapled peptides displayed more α-helix content than JMAlb or JMAlb but less than the analogous stapled molecules. The ellipticity values at 222 nm (ε222) of E1U, E2U, E4U, T1U, and T4U all fall between −9000 and −15 700 deg cm2 dmol−1 with E4U at the low (less structured) end and E2U at the high (more structured) end.

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and E4\text{U} at the high (more structured) end (Figures 2B and S3). The values reported for the analogous stapled molecules range from $-15,600$ to $-20,700$ deg cm$^2$ dmol$^{-1}$. Like the stapled variants, the $\varepsilon_{222}$ values of the unstapled peptides increased little if at all in the 25 and 100 $\mu$M concentration range (Figure S3), suggesting that all are predominantly monomeric at the lower concentrations employed (1 to 10 $\mu$M).

Next we made use of five cell lines to evaluate the extent to which each E1$^S$ variant modulated the viability of EGFR-dependent cells. Four of the five cell lines express EGFR but differ in the EGFR mutational state; one line does not express EGFR (Figure 3). 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$^-$ but not EGFR. The dose response curves in Figures 3 and S4 reveal several trends. First, as expected, cells expressing WT EGFR (A431) are sensitive to the small molecule tyrosine kinase inhibitor Gefitinib$^1$ and to the stapled peptides E1$^S$ and (less so) E2$^S$, but not the stapled peptides E4$^S$, T1$^S$, and T4$^S$, even at concentrations as high as 100 $\mu$M. Notably, the dose–response curves for the unstapled versions of E1$^S$ and E2$^S$ (E1$^U$ and E2$^U$, respectively) are superimposable on those for the analogous staple molecules. In fact, even the dose–response curves for the (virtually) inactive, stapled molecules (E4$^S$ and T4$^S$) are superimposable on the analogous unstapled variants (E4$^U$ and T4$^U$). The similarity in activity between stapled and unstapled analogs is especially surprising since the former are expected to possess longer half-lives in cellulo than the latter.$^{12,13}$ It is notable that the only sequence whose stapled and unstapled analogs behave differently is T1, where the staple replaces the leucine-rich interface required for formation of the proposed peptide·JM coiled coil.

The similarity between the effects of stapled and unstapled analogs is also apparent in H2030 and H1975 cells (Figure S4A): E1$^S$ and E1$^U$ are equipotent, as are E2$^S$ and E2$^U$. The EGFR, whereas H3255 and H1975 cells express single (L858R) or double (L858R/T790M) mutant forms, respectively; SK-N-MC cells express ErbB2$^-$ but not EGFR.$^{10}$

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The similarity between the effects of stapled and unstapled analogs is also apparent in H2030 and H1975 cells (Figure S4A): E1$^S$ and E1$^U$ are equipotent, as are E2$^S$ and E2$^U$. The
only instance where a stapled peptide and its unstacked analog perform differently occurs in H3255 cells that express L858R EGFR, a constitutively active EGFR mutant that is sensitive to gefitinib and erlotinib. H3255 cells are 2-fold more sensitive to E1S than to E1U, perhaps because of mutation-induced differences in JM structure in these receptor variants. Although previous reports might predict that the unstacked analog of an active, stapled inhibitor would show diminished activity,7,14 we find that E1S and E1U have nearly identical effects on the viability of these five cell lines.

We also evaluated the activity of three, new, stapled peptide variants of E1S and E2S. These molecules (E2.3S, E2.2S, and T4.2S) were chosen to provide additional information about the contribution of staple placement to inhibitor potency (Figure S4B–D). E2.3S, which like E1S carries an i to i + 7 cross-link on the helix face opposite that required for coiled coil formation, is inactive in all cell lines examined, whereas E2.2S and T4.2S are active at only the highest concentrations examined (IC₅₀ > 100 μM) and equally active in SK-N-MC cells that do not express EGFR (Figure S4A). Taken as a whole, the lack of activity displayed by E2.3S, E2.2S, and T4.2S indicates that position “i” of the heptad repeat is privileged with respect to inhibiting EGFR in these cell lines. This observation may reflect the requirement for multiple α-helix faces or binding modes; further work on this front is in progress.

In our previous work, we performed immunoblotting experiments to monitor the effect of each stapled peptide on the phosphorylation of EGFR and the downstream factors Akt and Erk in A431 cells.7 The stapled peptide E1S caused a dose-dependent decrease in EGFR autophosphorylation at several positions within the C-terminal tail. E1S inhibited phosphorylation at Y845, Y1045, Y1086, and Y1173, but not Y1068 and Y1148. A431 cells treated with E1S also showed decreased levels of phospho-Akt and phospho-Erk, whereas the levels of EGFR, Akt, and Erk in A431 cells were treated with 10 ng/mL EGF, and then lysed, immunoblotted, and visualized. Plots show the decrease in intensity of the indicated phospho-protein band relative to untreated cells. Error bars represent the standard error of the mean over at least four trials. Immunoblots of A431 cells treated with E2.2S, T4.2S, and E2.3S are found in Figure S5.

EGFR alone led to the expected increase in ReAsH fluorescence at the cell surface; this increase was unchanged by the presence of JMAlb. However, treatment of cells with 1 μM E1S or E1U led to a significant loss in ReAsH fluorescence. Neither E1S nor E1U affected ReAsH fluorescence in the absence of EGF (Figure 5). We conclude that E1S, like E1S, inhibits the intradimer coiled coil required for assembly of the active asymmetric kinase dimer. Like E1S, E1U is an allosteric inhibitor of EGFR. Experiments to identify the precise binding site(s) of E1S and E1U are in progress and will be reported in due course.

Figure 5. Comparison of the effects of E1S and E1U on formation of the EGFR-induced coiled coil within the EGFR JM using TIRF-M and bipartite tetracysteine display. CHO-K1 cells were transfected with plasmid encoding EGFR CCGT-1, treated with 1 μM of the indicated ligand for 1 h, stimulated in the presence or absence of 100 ng/mL EGF for 30 min, and labeled with ReAsH. The plot illustrates the change in ReAsH fluorescence at 568 nm of n CHO-K1 cells relative to the level of EGFR expression. Error bars represent the standard error of the mean. **p < 0.01, ****p < 0.0001; one-way ANOVA with Bonferroni postanalysis accounting for multiple comparisons.

Figure 4. Comparison of the effects of E1S and E1U on EGFR autophosphorylation and on phosphorylation of Akt and Erk1/2. A431 cells were treated with 10 μM of either E1S or E1U for 1 h, stimulated with 10 ng/mL EGF, and then lysed, immunoblotted, and visualized. Plots show the decrease in intensity of the indicated phospho-protein band relative to untreated cells. Error bars represent the standard error of the mean over at least four trials. Immunoblots of A431 cells treated with E2.2S, T4.2S, and E2.3S are found in Figure S5.

** ASSOCIATED CONTENT

Supporting Information

Experimental procedures and data. 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.

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