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The role of olefin geometry in the activity of hydrocarbon stapled peptides targeting eukaryotic translation initiation factor 4E (eIF4E)

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Abstract

Hydrocarbon stapled (HCS) peptides are a class of cross-linked α -helix mimetics. The technology relies on the use of α,α' -disubstituted alkenyl amino acids, which fully constrain the helical region to typically yield peptides with enhanced structural ordering and biological activity. Recently, monosubstituted alkenyl amino acids were disclosed for peptide stapling; however, the impact that this tether has on HCS peptide structure and activity has not yet been fully explored. By applying this HCS to the disordered peptide eIF4E-binding protein 1 (4E-BP1), we discovered that this type of tethering has a dramatic effect on olefin geometry and activity of the resultant stapled peptides, where the putative *trans* isomer was found to exhibit enhanced *in vitro* and cellular inhibitory activity against eIF4E protein-protein interactions. We further demonstrated that the metathesis catalyst used for ring-closing metathesis can influence monosubstituted HCS peptide activity, presumably through alteration of the *cis/trans* olefin ratio. This study represents one of the first in-depth analyses of olefin isomers of a stapled peptide and highlights an additional feature for medicinal chemistry optimization of this class of peptide-based probes.

Introduction

Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) functions as the primary gatekeeper of cap-dependent mRNA translation via its ability to bind and inhibit eIF4E, the m⁷GpppX-cap-binding translation initiation factor.^{1, 2} 4E-BP1 is an intrinsically disordered protein;³ yet, its eIF4E-binding domain (Asp55–Arg63) folds into a short α -helical structure upon association with eIF4E, thereby forming the “hot spot” of the protein-protein interaction (PPI) (Figure 1).^{4, 5} From the X-ray crystal structure of eIF4E bound to 4E-BP1,^{4, 6} the interface is flat with no distinct pockets for small molecule targeting. In fact,

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Conflicts of interest

There are no conflicts to declare.

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a previous high-throughput screen seeking to discover small molecule inhibitors of eIF4E PPIs led to the identification of an allosteric modulator.^{7, 8} Although peptides comprising the hot spot have been previously shown to exhibit binding affinities similar to that of the full-length 4E-BP1 protein, they exhibited little helical structure in solution⁴ and poor cellular activity in the absence of conjugation to a delivery vehicle.^{9–11} Because eIF4E recognizes 4E-BP1 via a helical conformation, we hypothesized that stabilization of this motif would favor enhanced eIF4E binding and pharmacological effects. Thus, our laboratory became interested in the development of constrained helical mimetics of 4E-BP1.

Hydrocarbon stapling (HCS) of α -helical peptides has emerged as a promising strategy towards translating peptide-based inhibitors of PPIs into the clinic.^{12–14} We previously disclosed **HCS-4E-BP1** that showed improved binding affinity and *in vitro* and cellular inhibitory activity over the linear peptide sequence.¹⁵ In this case, we utilized an α,α' -disubstituted alkenyl amino acid for stapling; however, monosubstituted alkenyl amino acids have also been reported.¹⁶ Few examples have been disclosed which utilize this type of HCS, and its impact on activity has been shown to be mixed, with the resultant stapled peptides either performing comparably¹⁷ or showing improved binding and inhibition in comparison to the disubstituted analogues.¹⁸ Based on its lack of α -methylation at the staple sites, we hypothesized that the resulting peptide would exhibit greater backbone flexibility within the helical region. Because the eIF4E-binding region of 4E-BP1 undergoes a disorder-to-order transition upon binding to eIF4E, we were eager to investigate the effects of monosubstituted stapling in our development of HCS 4E-BP1 peptides as chemical probes for interrogating the role of aberrant cap-dependent translation in human diseases.^{19, 20} Herein we disclose our efforts toward this goal and reveal the impact that alkenyl amino acid substitution can play in stapled peptide development.

Results and Discussion

Synthesis and Spectroscopic Characterization.

The monosubstituted HCS-4E-BP1 peptide (**mHCS-4E-BP1**) was synthesized via Fmoc solid-phase peptide synthesis as a C-terminal amide (Table 1). Fmoc-(*S*)-2-(4'-pentenyl)glycine was incorporated at the stapling sites and macrocyclization was subsequently performed using Grubbs I-catalyzed ring-closing metathesis (RCM). Lys57 and Glu61 were chosen as solvent-exposed staple sites of the *i, i+4* helix (Figure 1) based on our previous work.¹⁵ The peptide was acetylated at the N-terminus and peptide stock concentration was determined via amino acid analysis.

As preliminary characterization of this peptide, we measured its helicity via circular dichroism (CD) spectroscopy in comparison to 4E-BP1 and **HCS-4E-BP1**. Interestingly, unlike **HCS-4E-BP1**,¹⁵ **mHCS-4E-BP1** exhibited helicity values similar to the linear peptide (Figure 2). Its helical propensity, as measured in helix-inducing solvent 2,2,2-trifluoroethanol (TFE), was also comparable to that of 4E-BP1. Thus, the helix stabilizing properties of the mono- and disubstituted amino acids are clearly different within the context of the disordered 4E-BP1 sequence.

Direct Binding Studies.

We next measured binding kinetics and affinities via surface plasmon resonance (SPR) spectroscopy. eIF4E was immobilized via its N-terminal His₁₀ tag onto a HisCap sensor chip. Peptide was then flowed over the immobilized eIF4E protein. The association (k_a) and dissociation (k_d) rates and binding constant (K_d) were obtained by fitting to a 1:1 interaction model as previously described (sensorgram can be found in Figure S1).¹⁵ Again, **mHCS-4E-BP1**, unlike **HCS-4E-BP1**,¹⁵ showed no improvement over 4E-BP1 (Table 2). Despite exhibiting a 2.0-fold decrease in k_d , an almost equivalent loss in k_a resulted in a peptide with no net gain in affinity over the linear sequence. Coupled with our CD data, this indicates that **mHCS-4E-BP1** was not stapled into an eIF4E bioactive conformation and must undergo additional reorganization upon binding, which is analogous to what occurs with the disordered 4E-BP1 peptide.

In Vitro and Cellular Inhibitory Activity.

Finally, we measured inhibition of eIF4E PPIs, both *in vitro* and in cells. For our biochemical measurement, we utilized our PPI catalytic enzyme-linked click chemistry assay (cat-ELCCA) for the eIF4E–4E-BP1 PPI, which is a chemiluminescence-based PPI assay.²¹ As shown in Figure 3, the observed trends in CD and K_d also translated to *in vitro* inhibitory activity, and **mHCS-4E-BP1** exhibited a similar range of IC₅₀ value to 4E-BP1, while **HCS-4E-BP1** exhibited a ~7-fold enhancement.¹⁵

To test the inhibitory effect on cellular eIF4E PPIs, we used a m⁷GDP cap affinity chromatography assay, which captures eIF4E and its protein binding partners, 4E-BP1 and eIF4G.^{22, 23} Like 4E-BP1, eIF4G forms a similar canonical helix upon binding to eIF4E and competes for the same binding site; however, upon eIF4G binding, cap-dependent translation is initiated.^{4, 24} MDA-MB-231 triple negative breast cancer cells, which exhibit hyperactivated cap-dependent translation,²⁵ were treated with varying concentrations of either the linear or stapled 4E-BP1 peptides, and lysates were subjected to affinity purification and Western blot. Contrary to the biophysical and biochemical similarities between **mHCS-4E-BP1** and 4E-BP1, it exhibited dose-responsive inhibitory activity of eIF4E binding to eIF4G and 4E-BP1 similar to that of **HCS-4E-BP1**,¹⁵ while 4E-BP1 showed modest inhibition only at the highest concentration tested (Figure 4A). We then extended this analysis to two additional cell lines with enhanced cap-dependent translation, HCT116 colorectal carcinoma cells²⁶ and H1299 non-small cell lung cancer cells.²⁷ Similar to the results observed in MDA-MB-231 cells, both stapled peptides showed activity in antagonizing eIF4E PPIs with **HCS-4E-BP1** showing slightly enhanced inhibition in both MDA-MB-231 and HCT116 cells (Figure 4B, 4C). As the concentrations used well exceed the K_d and IC₅₀ values for these peptides, the comparable activities of the stapled peptides in cells was expected.

Evaluation of mHCS-4E-BP1 Olefin Isomers.

During the synthesis of **mHCS-4E-BP1**, we consistently isolated ~1:1 mixtures of two peaks showing identical masses, which we presumed were *cis* and *trans* olefin isomers;^{18, 28, 29} however, based on their very close retention times (Figure S2A), we generally combined all fractions with the appropriate molecular weight. Based on our results with the

mixture of **mHCS-4E-BP1**, we became interested in examining the activity of each of these isomers. After extensive HPLC purification, the two peptides were isolated, which we referred to as **mHCS-4E-BP1-i** and **mHCS-4E-BP1-ii** (Figure S2B). Based on NMR chemical shifts, we confirmed that these peptides were olefin isomers; however, due to small isolated quantities, the flexibility of the peptide and limited solubility, we could not measure accurate coupling constants to assign olefin geometry (Figure S3). Using a truncated **mHCS-4E-BP1** analogue containing amino acids Arg51–Arg63 (**trunc-mHCS-4E-BP1**), we were able to assign the **trunc-mHCS-4E-BP1-i** and **-ii** isomers as *trans* and *cis*, respectively, based on the measured coupling constants of the olefinic protons (Figure S4). Thus, based on the known preference of RCM for formation of the *trans* isomer,^{30, 31} we have putatively assigned **mHCS-4E-BP1-i** and **mHCS-4E-BP1-ii** as *trans* and *cis* in line with these results.

We first measured the helicity of the isomers via CD spectroscopy. Interestingly, both were more helical than the mixture **mHCS-4E-BP1** (12%, Figure 2) with average helicities of 23% and 26%, respectively (Figures 5A and 5C). While each exhibited enhanced helicity over 4E-BP1 (16%; Figure 2), neither was as ordered as **HCS-4E-BP1** (44%; Figure 2),¹⁵ confirming the unique properties of using mono- and disubstituted alkenyl amino acids for stapling the disordered 4E-BP1 peptide. Because both peptides exhibited helicities greater than that of **mHCS-4E-BP1**, we suspect that the isomers may self-assemble in aqueous solutions yielding decreased structural ordering within the mixture.³² In fact, preliminary analysis via dynamic light scattering (DLS) showed the formation of aggregates at higher aqueous concentrations of **mHCS-4E-BP1** (Figure S5).

We next measured IC₅₀ values using PPI cat-ELCCA. Surprisingly, and despite its almost equivalent helicity, **mHCS-4E-BP1-i** exhibited more potent activity with an average IC₅₀ value of 16 nM, 3.6-fold lower than that of **mHCS-4E-BP1-ii** (58 nM) (Figures 5B and 5C) and 1.9-fold lower than that of mixture **mHCS-4E-BP1** (31 nM) (Figure 3). This also extended to cells, and **mHCS-4E-BP1-i** showed enhanced antagonism amongst this series (Figure 6). Thus, these results indicate that olefin geometry plays a role in the inhibitory activity against eIF4E PPIs.

We then measured K_d via SPR to determine if binding kinetics could provide more insight into this interesting finding. Interestingly, the binding affinity of **mHCS-4E-BP1-i** was very similar to that **mHCS-4E-BP1-ii** with K_d values of 16 and 22 nM, respectively (Table 3; sensorgrams can be found in Figure S5). In comparison to the mixture, this corresponded to a 1.5-fold and 1.1-fold decrease, respectively (Tables 2 and 3). Both isomers exhibited slower association rates than **mHCS-4E-BP1**, but also slower dissociation rates (Tables 2 and 3). In fact, the *k_d* values were closer to that of **HCS-4E-BP1**¹⁵ (Tables 2 and 3) demonstrating a correlation between binding and dissociation as we previously observed with eIF4E-targeted stapled peptides.¹⁵ Because major kinetic differences were not observed between the isomers, we hypothesize that olefin geometry within the staple may cause minor long-range conformational changes within the peptides promoting differential inhibitory activity.

Investigation of the Effect of Metathesis Catalyst on mHCS-4E-BP1 Activity.

Because the isolation of **mHCS-4E-BP1-i** and **mHCS-4E-BP1-ii** was quite tedious, we were eager to investigate the effect of alternative metathesis catalysts in the RCM. Based on previous work by Grubbs and co-workers, the use of Grubbs II catalyst was found to promote higher stereoselectivity in RCM favoring the *trans* isomer.^{30, 31} Thus, we prepared **mHCS-4E-BP1** using this catalyst for stapling, which resolved as a unique single peak in comparison to the Grubbs I sample (Figure S7). Importantly, we observed greater helicity and *in vitro* inhibition (average IC₅₀ value of 14 nM) (Figure 7) and an improved K_d with this peptide (Table 4; sensorgram in Figure S8), further highlighting the impact that olefin geometry plays in the activity of **mHCS-4E-BP1**. In fact, the properties of the Grubbs II peptide were nearly identical to that of the more potent **mHCS-4E-BP1-i**. Interestingly, when we synthesized **HCS-4E-BP1**, which contains pentenylalanine amino acids, using Grubbs II, a peptide with equipotent activity resulted (Figure S9). Thus, these results demonstrate the unique properties of the pentenylglycine monomer and its resultant impact on olefin geometry and stapled peptide activity.

Conclusions

From this investigation, we revealed that in the case of 4E-BP1, monosubstituted stapling yielded a peptide with diminished helicity and inhibitory activity and weaker binding affinity in comparison to the α,α' -disubstituted **HCS-4E-BP1**, indicating that stabilization of a highly helical bioactive conformation is critical for potent eIF4E binding activity. Like other studies using monosubstituted cross-linking,^{18, 33} we also discovered that both *cis* and *trans* olefin isomers were produced during RCM. While this is common for longer staples, particularly of *i, i+7* helices,^{28, 29, 34} few instances have been reported for *i, i+4* helices.^{18, 31} Here we described one of the first in-depth analyses of olefin isomers of a stapled peptide. Interestingly, we found that the putative *trans* isomer exhibited better activity than the *cis* both *in vitro* and in cells. Future efforts will be focused on deciphering the mechanism of this enhanced activity, whether it be through differential binding to eIF4E, cellular penetrability or proteolytic stability. By utilizing the more *trans*-selective Grubbs II catalyst,³⁰ we further demonstrated the significant role that olefin geometry plays in the activity of this peptide. While we could not assign the olefin geometry in **HCS-4E-BP1** (data not shown), presumably due to its flexibility, that of **sTIP-04**, another eIF4E-binding stapled peptide,^{15, 35} was resolved as the *cis* isomer (Figure S10), which is typical for those containing alkenylalanine amino acids.^{17, 18, 32, 36–38} Thus, future efforts in the field should be devoted towards the exploration of new metathesis catalysts with higher selectivity and metathesis-based methodologies^{31, 39} to enable the synthesis of pure olefin stereoisomers, particularly in the cases of disordered peptide substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1. Interface of the eIF4E-4E-BP1 PPI (PDB: 4UED). The amino acids used as the staple sites (Lys57 and Glu61) are highlighted.

Fig. 2.

Peptide helicities as determined via CD spectroscopy in 5 mM sodium phosphate buffer, pH 7.4 at 25 °C. (A) Spectra. (B) Table of average helicity values measured at 25–100 μ M across the concentrations tested. For the TFE experiments, only 100 μ M peptide was used at 40% TFE.

Fig. 3.

In vitro inhibitory activities of 4E-BP1, **HCS-4E-BP1**¹⁵ and **mHCS-4E-BP1** as determined via PPI cat-ELCCA. (A) IC₅₀ curves. (B) Table of IC₅₀ values presented as 95% confidence intervals.

Fig. 4.

Cellular inhibitory activities of 4E-BP1, **mHCS-4E-BP1** and **HCS-4E-BP1**¹⁵ peptides as determined via m⁷GDP cap affinity chromatography. Inhibition of the eIF4E–4E-BP1 and eIF4E–eIF4G PPIs in (A) MDA-MB-231 cells, (B) HCT116 cells (2.5 μ M), and (C) H1299 cells (2.5 μ M). In all cases, cells were grown in 10% FBS and treated for 6 h; eIF4E was used as a normalization control.

Fig. 5. Characterization of **mHCS-4E-BP1-i** and **mHCS-4E-BP1-ii**. (A) Peptide helicity spectra in 5 mM sodium phosphate buffer, pH 7.4 at 25 °C. (B) IC₅₀ curves from PPI cat-ELCCA. (C) Tabulated data from (A) and (B).

Fig. 6. Cellular inhibitory activities of **mHCS-4E-BP1** and its isomers as determined via m^7 GDP cap affinity chromatography in MDA-MB-231 cells.

Fig. 7.

Characterization of **mHCS-4E-BP1** peptides prepared using different Grubbs catalysts. (A) Peptide helicity spectra in 5 mM sodium phosphate buffer, pH 7.4 at 25 °C. (B) IC₅₀ curves from PPI cat-ELCCA. (C) Tabulated data from (A) and (B).

Table 1.

Peptides used within this study

Peptide	Sequence
4E-BP1	AcNH- ⁴⁹ G T R I I Y D R K F L M E C R N ⁶⁴ -CONH ₂

HCS-4E-BP1	AcNH- ⁴⁹ G T R I I Y D R * F L M * C R N ⁶⁴ -CONH ₂

mHCS-4E-BP1	AcNH- ⁴⁹ G T R I I Y D R # F L M # C R N ⁶⁴ -CONH ₂

* = (S)-pentenylalanine;

= (S)-pentenylglycine

Table 2.SPR Analysis of 4E-BP1, **HCS-4E-BP1¹⁵** and **mHCS-4E-BP1** Peptides at 25 °C

Peptide	$k_a \times 10^6$ (M ⁻¹ s ⁻¹)	$k_d \times 10^{-3}$ (s ⁻¹)	K_d (nM)
4E-BP1	0.82 ± 0.3	21 ± 4	26 ± 6
HCS-4E-BP1	1.5 ± 0.2	5.3 ± 4	4.1 ± 3
mHCS-4E-BP1	0.47 ± 0.2	11 ± 2	24 ± 1

Table 3.SPR Analysis of **mHCS-4E-BP1** Isomers at 25 °C

Peptide	$k_a \times 10^6$ (M ⁻¹ s ⁻¹)	$k_d \times 10^{-3}$ (s ⁻¹)	K_d (nM)
mHCS-4E-BP1-i	0.38 ± 0.1	6.1 ± 0.1	16 ± 1
mHCS-4E-BP1-ii	0.32 ± 0.3	6.9 ± 2	22 ± 2

Table 4.SPR Analysis of **mHCS-4E-BP1** Peptides Prepared Using Different Grubbs Catalysts at 25 °C

Peptide	$k_a \times 10^6$ (M ⁻¹ s ⁻¹)	$k_d \times 10^{-3}$ (s ⁻¹)	K_d (nM)
mHCS-4E-BP1 (Grubbs I)	0.47 ± 0.2	11 ± 2	24 ± 1
mHCS-4E-BP1 (Grubbs II)	0.37 ± 0.1	7.1 ± 1	19 ± 1