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Novel all-hydrocarbon stapled p110 α [E545K] peptides as blockers of the oncogenic p110 α [E545K]-IRS1 interaction

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Abstract

To follow up on our recent discovery of the 18-amino acid all-hydrocarbon [i, i+4]-stapled p110 α [E545K] peptide **1** that was shown to potently block the intracellular p110 α [E545K]-IRS1 interaction (a protein-protein interaction uniquely present in cancer cells expressing p110 α [E545K]) and the growth of the xenograft tumors formed by cancers harboring this mutation, in the current study we prepared and examined six derivatives of **1**, i.e. stapled peptides **2-A**, **2-B**, **3-A**, **3-B**, **4-A**, **4-B**. We found that **2-A**, **2-B**, **4-A**, and **4-B** had higher % α -helicity than **1**; moreover, the enhanced % α -helicity also led to an enhanced proteolytic stability. When compared with **1**, the structurally simplified 14-amino acid **4-A** and **4-B** were found to more potently deactivate the AKT phosphorylation at Ser473 in the p110 α [E545K]-expressing colon cancer cells, whose activation was previously demonstrated by us to be specifically derived from the p110 α [E545K]-IRS1 interaction. The preliminary findings from the current study have laid a foundation for future more extensive studies on the stapled p110 α [E545K] peptides newly identified in the current study.

Graphical abstract

All-hydrocarbon [i, i+7]-stapled p110 α [E545K] peptides **4-A** and **4-B** were found in the current study to possess greater % α -helicity and proteolytic stability than the all-hydrocarbon [i, i+4]-stapled p110 α [E545K] peptide **1**, the first chemical blocker discovered recently by us for the oncogenic p110 α [E545K]-IRS1 interaction uniquely present in the colon cancer cells expressing p110 α [E545K] instead of the wild-type p110 α . Preliminary cellular study with such colon cancer cells showed that **4-A** and **4-B** were stronger deactivators of the p110 α [E545K]-IRS1 interaction-initiated AKT phosphorylation at Ser473 than **1**.

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Keywords

protein-protein interaction; p110α[E545K]; IRS1; stapled peptide; % α-helicity; proteolytic stability; AKT phosphorylation; anti-cancer therapeutic

We recently found that the catalytic subunit of phosphatidylinositol 3-kinase α (PI3Kα) harboring the high frequent oncogenic E545K mutation at its helical domain (i.e. p110α[E545K]) incurred a direct interaction with insulin receptor substrate 1 (IRS1) independent of the regulatory subunit p85α of PI3Kα.¹ The p110α[E545K]-IRS1 interaction was found to play a critical role in promoting the growth of the colon cancer cells expressing p110α[E545K]. Moreover, the 18-amino acid all-hydrocarbon [i, i+4]-stapled peptide **1** instead of its linear counterpart **1a** (Figure 1) was found in the same study to potently block the intracellular p110α[E545K]-IRS1 interaction and the growth of the xenograft tumor formed by the p110α[E545K]-expressing colon cancer cells when **1** was injected directly into the tumor. The effectiveness of compound **1** could have resulted from the ability of its intra-chain all-hydrocarbon macrocycle (staple) to induce and stabilize an α-helical conformation, which would also lend a support for the possibility that the p110α[E545K]-IRS1 interaction would also be mediated by an α-helix on p110α[E545K], like that for the normal association of p110α with p85α.² However, the % α-helicity and proteolytic stability of compound **1** are not high, as indicated in Figures 2 and 3, and therefore need to be improved. An enhanced % α-helicity and proteolytic stability may also increase the inhibitory potency toward the intracellular p110α[E545K]-IRS1 interaction and the corresponding colon cancer cell growth. In the current study, we performed a preliminary structure-activity-relationship study on compound **1** through the preparation and examination of its derivative stapled peptides **2-A**, **2-B**, **3-A**, **3-B**, **4-A**, and **4-B** (Figure 1). It should be noted that the size and double bond position of a macrocycle as well as the stereochemistry of the relevant α-carbon of the [i, i+4]-stapling and [i, i+7]-stapling shown in Figure 1 are those already optimized and employed previously by other researchers.⁴⁻⁷

We reasoned that the % α-helicity and proteolytic stability of compound **1** could be enhanced by trimming the loose N- and/or C-terms of this stapled peptide. While a 3-dimensional structural determination could reveal the boundary of the α-helix and the adjacent disordered end(s) in compound **1**, in the current study we invoked a simple liquid chromatography (LC)-mass spectrometric (MS) analysis of the proteolytic digestion mixture of compound **1**. Specifically, we looked for and determined the m/z value of the high performance liquid chromatography (HPLC) peak that became persistent with the proteolytic digestion time. From the m/z measurement, we were then able to ascertain the sequence of the digestion product which would correspond to the α-helix part of compound **1** without loose ends. From such analysis, the 10-mer [i, i+4]-stapled peptidic structure **5** was deduced, as shown in Figure 4. However, it is clear that this structure does not include the key K545 residue and K545 seems to locate by the end of the α-helix which was induced and stabilized by the particular intra-chain all-hydrocarbon macrocycle of compound **1**.

Therefore, we turned our attention to the [i, i+7]-stapled derivatives of compound **1** since this stapling system is more expansive than the [i, i+4]-stapling system so hopefully the key residue K545 would be included in the stabilized α -helix. It should be noted that pursuing other [i, i+4]-stapled derivatives of compound **1** that would potentially include K545 in the α -helix, by shifting the macrocycle attaching point(s), would be unrealistic due to the limited number of amino acid residues in compound **1** and the necessity to maintain the macrocycle on the same face of α -helix. By examining the amino acid sequence of compound **1**, two modes of [i, i+7]-stapling would be possible for maintaining the macrocycle on the same face of α -helix. Accordingly, compounds **2** and **3** were designed (Figure 1). Of note, the wavy line in the [i, i+7] macrocycle in the chemical structures of compounds **2** and **3** denotes the occurrence of the *cis* and *trans* olefinic geometric isomers in a molar ratio of roughly 1:1 during our compound preparation. These isomers were designated as **2-A**, **2-B**, **3-A**, and **3-B**.

Compounds **2** and **3** were synthesized according to Scheme 1 *via* the solid-phase peptide synthesis (SPPS) followed by the on-resin ruthenium-catalyzed ring closing metathesis (RCM) and purified by the reversed-phase (RP)-HPLC, in the same manner as compound **1** according to our previously published procedures.¹ By using RP-HPLC, the olefinic geometric isomers **2-A**, **2-B**, **3-A**, and **3-B** were all isolated, and their exact masses were confirmed with MS analysis (Table 1).

With these compounds in hand, they were subjected to circular dichroism (CD) determination and a proteolytic digestion assay. As indicated in Figures 2 and 3, the purified compounds **3-A** and **3-B** were both found to have lower % α -helicity and proteolytic stability than compound **1**; however, the purified compounds **2-A** and **2-B**, especially **2-A**, exhibited higher % α -helicity and proteolytic stability than compound **1**. Therefore, compound **2-A** was selected as the template for the structural simplification, as described above for compound **1**. When **2-A** was subjected to proteolytic digestion, followed by HPLC analysis of the digestion mixture and mass spectral determination of the persistent HPLC peak, the 14-mer [i, i+7]-stapled peptidic structure **6** was deduced, as shown in Figure 4. It is clear that this structure does include the key K545 residue within the α -helix which was induced and stabilized by the particular intra-chain all-hydrocarbon macrocycle of compound **2-A**. Therefore, we decided to prepare compound **4** (Figure 1). Again, the wavy line in the [i, i+7] macrocycle in the chemical structure of compound **4** denotes the occurrence of the *cis* and *trans* olefinic geometric isomers in a molar ratio of roughly 1:1 during our compound preparation. Compound **4** was synthesized in the same manner as compounds **2** and **3** according to Scheme 1. By using RP-HPLC, the olefinic geometric isomers **4-A** and **4-B** were both isolated, and their exact masses were also confirmed with MS analysis (Table 1).

After they were subjected to CD determination and a proteolytic digestion assay, the purified compounds **4-A** and **4-B** were found to largely have comparable % α -helicity and proteolytic stability to those of compound **2-A** (Figures 2 and 3), despite **4-A** was proteolytically more stable than another two.

Taken together, the above findings are consistent with the notion that proteolytic stability of a stapled peptide can be improved by increasing its % α -helicity.⁴

Next, we were interested in preliminarily assessing whether the structurally simplified **4-A** and **4-B** were able to cross the cell membrane and act on intracellular signaling initiated by the p110 α [E545K]-IRS1 interaction. As demonstrated previously by us¹ and schematically illustrated in Figure 5, an activated phosphorylation of AKT at Ser473 can be specifically derived from the p110 α [E545K]-IRS1 interaction in the p110 α [E545K]-expressing colon cancer cells; therefore, a blockade of the p110 α [E545K]-IRS1 interaction would deactivate this AKT phosphorylation. After incubated with the DLD1 human colon cancer cells expressing p110 α [E545K] instead of the wild-type p110 α , stapled peptides **4-A** and **4-B** were found to be cell permeable and to potently deactivate the intracellular AKT phosphorylation at Ser473. As indicated by Western blotting analysis (Figure 6), **4-A** and **4-B** are actually stronger deactivators of the p110 α [E545K]-IRS1 interaction-initiated AKT phosphorylation at Ser473 than **1**. It should be noted that the cell permeability of these compounds could be attributed to a combination of their assuming α -helical conformation and their overall charge being 0 (for **4-A** and **4-B**) or +1 (for **1**) under physiological condition (indicated in Figure 1, within the ideal charge window (i.e. 0-2)⁴). Moreover, even though **4-A** has higher % α -helicity and is proteolytically more stable than **4-B**, this latter stapled peptide was observed to be a slightly stronger deactivator of AKT phosphorylation than **4-A**. We currently don't have an explanation for this, but one possible reason could be the more favorable membrane interaction of the macrocycle in **4-B** than that in **4-A**, leading to a higher membrane permeability for **4-B**; future work will be needed to evaluate this possibility.

We were also attempting to use proton magnetic resonance spectrometry (¹H-NMR) to assign the geometry of the double bonds respectively present in the macrocycles of **4-A** and **4-B**. It is known that it is possible to make this geometric assignment based on the magnitude of the coupling constant between the two protons across a 1,2-disubstituted double bond, with that of a *trans* double bond being greater than that of a *cis* double bond.¹⁰ In fact, this approach has been previously taken to assign the geometry of the intra-cycle double bonds in all-hydrocarbon stapled peptides.¹¹ By scrutinizing the chemical structures of **4-A** and **4-B**, the chemical shift values of the double bond protons would be at 5-6 ppm, a window free of the chemical shift values from all the remaining protons in both molecules if the ¹H-NMR experiment is run in a deuterated version of a protic solvent (e.g. CD₃OD) so that the ¹H-NMR spectrum will be free of the signals from the exchangeable protons (e.g. those on NH, OH, COOH) in a sample molecule,¹⁰ simplifying the data interpretation. However, after several attempts of the ¹H-NMR experiment in CD₃OD with either 400-MHz or 600-MHz NMR spectrometer, we were still unable to assign unambiguously the double bond geometry for **4-A** and **4-B**, due to the severe overlapping of the signals derived from the coupling among the two double bond protons and their neighboring CH₂ protons (data not shown). This inability of making an unambiguous assignment of the geometry for the intra-cycle double bonds in all-hydrocarbon stapled peptides **4-A** and **4-B** is analogous to the scenario reported previously in literature.¹² Therefore, future work is still needed to assign

the double bond geometry for **4-A** and **4-B**; to this end, one possible future experiment could be the 3-dimensional structural elucidation for these two stapled peptides.

The preliminary findings from the current study have laid a foundation for future more extensive studies on the stapled p110 α [E545K] peptides (**4-A** and **4-B** in particular) newly identified in the current study, with the goal of developing potent, cell permeable, and metabolically stable stapled p110 α [E545K] peptide blockers of the oncogenic p110 α [E545K]-IRS1 interaction as novel anti-colon cancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The chemical structures of the all-hydrocarbon stapled p110 α [E545K] peptide **1** and its derivative stapled peptides **2-A**, **2-B**, **3-A**, **3-B**, **4-A**, and **4-B**. **Note:** (i) The number in the bracket under a chemical structure denotes the overall charge of the corresponding stapled peptide under physiological condition. (ii) The two numbers in the parentheses under the chemical structure of a stapled peptide denote the two positions in the linear peptide **1a** (derived from the human p110 α [E545K]) picked for peptide stapling. (iii) The key residue K545 is indicated with an asterisk. (iv) While only one olefinic geometric isomer (arbitrarily designated as the *trans* isomer) was formed during our preparation of the [i, i+4]-stapled peptide **1**, *cis* and *trans* olefinic geometric isomers were both formed in a molar ratio of roughly 1:1 during our preparation of the [i, i+7]-stapled peptides **2**, **3**, and **4** (denoted with the wavy line, arbitrarily designated as **2-A/2-B**, **3-A/3-B**, and **4-A/4-B** with no olefinic geometry assigned yet). These observations are consistent with literature precedents.³

Figure 2.

The CD spectra of all the compounds shown in Figure 1 at 50 μ M and the corresponding % α -helicity values. The mean residue ellipticity $[\theta]$ and the % α -helicity were calculated according to literature equations.⁸

Figure 3.

The proteolytic stability of the linear peptide **1a** (▲) and the stapled peptides **1** (▲), **2-A** (◆), **2-B** (◆), **3-A** (■), **3-B** (■), **4-A** (●), and **4-B** (●), as assessed by the pronase digestion assay. The straight lines denote the degradation trends of different test compounds. Pronase was employed in the assay as the proteolytic enzyme preparation since it is a mixture of a variety of different types of proteases and peptidases and therefore has a very broad substrate specificity.⁹ See “Supplementary Material” for the assay details.

Figure 4.

The chemical structures of the 10-mer [i, i+4]-stapled peptidic structure **5** and the 14-mer [i, i+7]-stapled peptidic structure **6**, deduced from LC-MS analysis of the proteolytic digestion mixtures of compounds **1** and **2-A**, respectively. The wavy line in the [i, i+7] macrocycle in structure **6** denotes the un-assigned configuration for the corresponding olefinic geometric isomer derived from compound **2-A**.

Figure 5.

A schematic illustration of the activated AKT phosphorylation at Ser473 specifically derived from the p110 α [E545K]-IRS1 interaction in the p110 α [E545K]-expressing colon cancer cells, and the deactivation of this AKT phosphorylation following the blockade of the p110 α [E545K]-IRS1 interaction by an all-hydrocarbon stapled p110 α [E545K] peptide.

Figure 6.

The Western blotting analysis of the ability of stapled peptides **1**, **4-A**, and **4-B** (50 μ M each) to deactivate the AKT phosphorylation at Ser473 in DLD1 human colon cancer cells expressing p110 α [E545K] instead of the wild-type p110 α .

Scheme 1.

The solid phase synthesis of compounds **1**, **2-A**, **2-B**, **3-A**, **3-B**, **4-A**, and **4-B**. The ruthenium-containing Grubbs catalyst™ (1st generation) was used to catalyze the on-resin RCM. The synthetic scheme of compound **1** is also shown to illustrate that all the compounds were synthesized in the same manner. SPPS, solid-phase peptide synthesis; Ac, acetyl; Reagent K, a trifluoroacetic acid (TFA)-containing peptide cleavage cocktail.

Table 1MS analysis of compounds **2-A**, **2-B**, **3-A**, **3-B**, **4-A**, and **4-B**^a

Compound	Ionic Species	Calculated m/z's	Observed m/z's
2-A	[M+2H] ²⁺	1213.63	1213.95
	[M+3H] ³⁺	809.42	809.83
	[M+4H] ⁴⁺	607.32	607.63
2-B	[M+2H] ²⁺	1213.63	1213.88
	[M+3H] ³⁺	809.42	810.00
	[M+4H] ⁴⁺	607.32	607.81
3-A	[M+2H] ²⁺	1195.13	1195.93
	[M+3H] ³⁺	797.09	797.57
	[M+4H] ⁴⁺	598.07	598.66
3-B	[M+2H] ²⁺	1195.13	1195.47
	[M+3H] ³⁺	797.09	797.48
	[M+4H] ⁴⁺	598.07	598.54
4-A	[M+H] ⁺	1936.08	1936.53
	[M+2H] ²⁺	968.55	968.80
	[M+3H] ³⁺	646.03	646.30
4-B	[M+H] ⁺	1936.08	1935.68
	[M+2H] ²⁺	968.55	968.70
	[M+3H] ³⁺	646.03	646.31

^a All compounds were measured with the positive ion mode of electrospray ionization. See "Supplementary Material" for a copy of the MS spectra.