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Stapling a G-quadruplex specific peptide

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Keywords: G-quadruplex; G-quadruplex-binding protein; RHAU helicase; stapled peptides.

ABSTRACT

G-quadruplexes (G4) are non-canonical four-stranded nucleic acid structures and the RHAU helicase has been identified to have high specificity for recognition of parallel-stranded G4. We have designed and synthesized two stapled peptide analogues of the G4-specific motif of RHAU, which preserve the binding to G4. Characterization of these peptides identified the stapled variants to exhibit higher helical formation propensity in aqueous buffer in comparison to the native RHAU sequence. Moreover, the stapled peptides exhibit superior enzymatic stability towards α -chymotrypsin. Our stapled RHAU peptides can serve as a new tool for targeting G4 nucleic acids structures.

INTRODUCTION

G-quadruplex (G4) is a unique four-stranded nucleic acid secondary structure composed of consecutively stacked G-tetrads. Each G-tetrad contains four guanine bases held in a square planar arrangement by Hoogsteen hydrogen bonding. The G4 is further stabilized by monovalent cations, such as Na^+ or K^+ , through metal ion coordination to the guanine carbonyl groups [1]. A diverse range of conformations has been observed for G4s and this polymorphic DNA structure can be generally classified into two major types: parallel (composed of unidirectional DNA/RNA strands) and non-parallel (comprising one of two strands aligned in opposing direction) G4s [2,3].

G4s can form in guanine-rich DNA and/or RNA sequences, such as the gene promoters, telomeres, human telomerase RNA and TERRA [2,4]. The formation of G4s within cells has been demonstrated via immunofluorescence techniques [5]. Bioinformatics analyses of the human genome have determined G4 forming sites to be highly prevalent [6] and often associated with vital cellular processes, such as DNA replication and transcription, RNA translation [7] and telomeric maintenance [8]. Several proteins such as Bloom syndrome protein (BLM) [9], Werner syndrome protein (WRN) [9], PIF1 helicase [10], and RHAU helicase [11] were identified to bind and unwind G4, with impaired version of some proteins associated with genetic diseases. In particular, the RHAU helicase has been shown to specifically bind [11] and unwind parallel G4 structures [12]. This 1008 amino acid-long protein from the DEAD/H box superfamily is associated with telomere maintenance [13], spermatogonia differentiation [14,15] and stress granule regulation [16–18]. Structural analysis by NMR spectroscopy and X-ray crystallography showed that human RHAU and the bovine RHAU homologue specifically recognize G4 structures using an α -helix formed at the N-terminal [19,20].

The α -helix represents the most prevalent secondary motif in proteins, with helical motifs often identified as the key structural element participating in important intracellular protein-protein interactions (PPIs) [21,22]. Although much effort has been invested to isolate biologically active helical motifs involved in PPIs, there remain challenges to their successful application as therapeutic agents as the isolated peptides exhibited high conformational and structural instability. The unfolding of the helical motifs into linear peptides resulted in poor cell permeability as well as high susceptibility to proteolytic degradation, rendering them unsuitable as therapeutic tools [23]. To stabilize the α -helical folds and prevent the peptide from unfolding, a stapling strategy

which connects selected helical twists with covalent linkages has been developed, where two amino acids on the same peptide face are substituted to facilitate formation of a covalent bridge from their side chains [24]. The resulting stapled peptides has been shown to exhibit robust stability profiles without major perturbation to their native conformation [25], with this approach leading to the increased application of stapled peptides for therapeutic applications [26].

Our previous work on RHAU has identified an α -helical 18-amino acid peptide containing the N-terminal RHAU-specific motif (*RHAU18*; Figure 1A) as an essential G4 binding motif primarily involving hydrophobic and electrostatic interactions at the peptide-G4 interface [27]. This peptide could potentially serve as a diagnostic tool for G4 detection or engineered to achieve gene expression regulation in cells. Correspondingly, there is a need to ensure the RHAU peptide remains structured in different physiochemical environment to preserve the peptide helicity and G4-recognition function, while increasing the peptide stability towards proteolytical degradation for use in cells.

In this study, we applied the stapling strategy to stabilize the RHAU peptide helical form and investigated the biophysical properties of the native RHAU helical motif in comparison to its two stapled analogues. A 23-amino acid RHAU peptide, *RHAU23*, which was slightly longer than *RHAU18* and displayed similar binding affinity to parallel G4 as *RHAU18* [19] is utilized in the stapled peptide design. Two stapled RHAU peptides, *RHAU LSAH₁* and *RHAU LSAH₂* involving different stapling sites are produced and analyzed (Figure 2, Table S1), demonstrating conservation of the α -helical fold and G4-binding ability. These stapled peptide analogues were found to exhibit higher resistivity toward protease degradation, with their robust nature facilitating the use of RHAU peptide as a potential tool towards identification of G4 structures in cells.

MATERIALS AND METHODS

Peptide synthesis

All RHAU peptides, including the linear and stapled analogues were prepared on an automated microwave peptide synthesizer (Biotage Initiator+ Alstra) using a Fmoc solid-phase peptide synthesis (SPPS) procedure with Rink Amide resin as the solid support. The peptides were cleaved off from the resin and globally deprotected with reagent K. Cold diethyl ether was added to

precipitate crude peptides. The crude peptides were purified by reverse phase HPLC and characterized by MALDI mass spectrometry to be >95 % pure. The final concentration of the peptide was determined using bicinchoninic acid assay and UV absorbance at 280 nm.

Circular dichroism (CD) spectroscopy

The linear and stapled peptides were dissolved in water to final concentration of 50 μ M. The CD spectra were obtained with 1 mm quartz cuvette on Jasco J715 spectrometer at 25 °C. The measurements were recorded for wavelength 190 – 260 nm and the data normalized by calculating the mean ellipticity (θ). The helicity of each peptide was calculated based on the molar ellipticity $[\theta]$, given in $\text{deg.cm}^2/(\text{dmol})$, of the peptide's spectrum at 222 nm and the number of amino acids in the peptide sequence as shown in the equation (1) below [28].

$$[\theta] = (\theta_{\text{raw}} * M) / (1000 * c * l) \quad (1)$$

where θ_{raw} is background corrected raw CD signal (degrees), l is the path length of the cuvette (mm), c is the concentration of the peptide (mg/mL), M is the mean residue molecular weight (g/mol).

Protease resistance

The peptides were reconstituted in buffer containing 50 mM Tris-HCl (pH 7.4) to final concentration of 1 mM. α -Chymotrypsin was dissolved in buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM CaCl_2 to final concentration of 5 ng/ μ L. In reaction solution of 100 μ L, the peptide was incubated with α -chymotrypsin at 37 °C (peptide/enzyme ratio 50:1). 20 μ L aliquots of the digestion mixture was taken at timepoint 0, 20, 48 and 72 h and the reaction stopped with the addition of 1 M HCl. The digested peptide fragments were analyzed by HPLC, monitoring at 280 nm to determine the amount of peptide degradation.

Native gel electrophoresis binding assay

In binding buffer containing 20 mM potassium phosphate (pH 6.8), 70 mM KCl, 30 μ M DNA was incubated with 7-fold peptide for 30 minutes at room temperature. 2 μ L of 50 % (v/v) glycerol was added to make up a total reaction volume of 20 μ L before resolving on 10 – 15 % polyacrylamide

gels (37.5:1) using 20 mM potassium phosphate running buffer. The gel was visualized using UV shadowing and processed using ImageJ [29].

RESULTS AND DISCUSSION

Design strategy of stapled RHAU peptides

In this study, the 23-aa peptide *RHAU23* [19] (Figure 2) was utilized as the template sequence to design the stapled RHAU peptides. The L-shaped α -helical conformation of the RHAU peptide is essential for G4-peptide interaction (Figure 1A), with the amino acids G7, I10, G11 and A15 involved in direct interaction with the G-tetrad face [19]. It is critical to retain these elements in the design of the stapled peptide to mimic the native peptide-DNA interaction.

The helical wheel presentation of the essential G4 binding motif of RHAU (Figure 1B) shows the G4 binding site of the RHAU peptide to be composed mostly of hydrophobic amino acids while the opposite face of the peptide is constituted mostly of polar residues. As illustrated in Figure 1B, the amino acids on the non-G4-interacting face of the RHAU peptide can be substituted to accommodate different stapling sites, with a total of two stapled constructs attempted in this study (Figure 2A). We have chosen lactam-bridge linkage as a stapling strategy as it exhibit near-native structure due to the natural amide bond composition and greater *in vivo* stability [30]. In addition, the chemistry involved in the stapling reaction (side chain protected with allyl protecting groups) is orthogonal to the synthesis of the main peptide chain (Fmoc chemistry) (Figure 2B).

To facilitate formation of the lactam bridge, two amino acids at i , $i+4$ positions on the same face of the native peptide sequence were substituted with glutamic acid (E) and lysine (K) respectively. In the case of RHAU alpha helix 1 (*RHAU AH₁*), arginine 8 (R8) and methionine 12 (M12) were substituted with E and K respectively to form the lactam-stapled *RHAU LSAH₁*. In the second stapled RHAU analogue, the tryptophan 13 (W13) is substituted to K, forming the non-stapled *RHAU AH₂* peptide and the stapled version *RHAU LSAH₂* (Figure 2A). All peptides were synthesized using an automated microwave synthesizer, purified by HPLC to achieve over 95 % purity, with characterization via MALDI mass spectrometry affirming the successful formation of the stapled peptides (Figure S1).

Binding of stapled RHAU peptides to parallel G4

The binding properties of the RHAU analogues to a parallel G4 DNA (*T95-2T*) (Figure 1A) were examined using native polyacrylamide gel electrophoresis. Previous studies established *RHAU23* to bind parallel G4s with affinity of $K_D \sim 1 \mu\text{M}$ [19]. From the gel analysis of *T95-2T* titrated with different RHAU peptides, band shifts corresponding to G4-RHAU complex formation were observed for the stapled analogues (*RHAU LSAH₁* and *RHAU LSAH₂*) as well as the non-stapled analogues. Majority of the G4 was complexed with the RHAU peptide derivatives with approximately 10 % of the G4 remaining unbound, while in the case of the native *RHAU23* peptide, the full G4-RHAU complex band was observed (Figure 3). Although the G4-binding capacity of these RHAU mutants appeared to be mostly preserved, the gel data suggested the use of R60E/M64K and W65K substitution to be slightly unfavorable for G4-binding. As no significant increase in band intensity corresponding to the free G4 was observed between the non-stapled and stapled RHAU variants, this indicated the placement of the lactam bridge within the RHAU mutants and the constrained helical conformation in the stapled peptides to not affect G4-binding interaction. Our gel data suggests the RHAU peptide to be adaptable to the stapling approach without overly compromising its G4-binding ability.

Stapled RHAU peptides displayed enhanced α -helical content

The secondary structure of *RHAU23* analogues was studied with CD spectroscopy in which the average α -helical content of the stapled peptides in solution was compared to their respective non-stapled analogues (Figure S2). Analysis of individual CD spectrum recorded for the RHAU peptides revealed all same-length non-stapled peptides to be unstructured in water (5 – 8 % α -helical content, respectively), whereas both stapled analogues *RHAU LSAH₁* and *RHAU LSAH₂* exhibited a 10-fold to 18-fold enhancement in α -helical content (95 % and 88 %, respectively) in aqueous solution. The increased α -helical content observed in the stapled peptides could be a result of the constrained peptide conformation introduced by the lactam bridge, with appropriate amino acids (*i* and *i+4*) held closer in space to promote secondary structure formation.

Stapled RHAU peptides are resistant to proteolytic degradation

To assess the protease stability of the stapled peptides, we measured their susceptibility towards α -chymotrypsin, a protease that predominantly cleaves at the carboxyl side of a positively charged amino acids such as tryptophan (W), tyrosine (Y) and leucine (L). These amino acids are present on both the native and mutated RHAU peptides used in this study. A time-dependent study over a period of 48 h was performed and the ratio of the remaining intact peptide to digested peptide was examined by analytical RP-HPLC (Figure 4). The non-stapled RHAU analogues *RHAU AH₁* and *RHAU AH₂* showed high susceptibility to α -chymotrypsin-mediated degradation, with approximately 60 % of the peptide cleaved following 48 h incubation at 37 °C. In sharp contrast, no elution peaks corresponding to the peptide degradation products were observed on the HPLC profile for both stapled peptides *RHAU LSAH₁* and *RHAU LSAH₂*. These results clearly demonstrated the inherent robustness of the stapled peptides over linear peptides with respect to proteolytic stability.

CONCLUSIONS

We have designed and synthesized stapled peptide analogues of the RHAU G4-specific motif by introducing the side-chain *i*, *i* + 4 lactam cyclization which successfully preserved the binding of the peptides towards parallel G4s. Moreover, significant enhancement to the peptide helicity content and superior stability against α -chymotrypsin digestion was observed for the stapled RHAU peptides. From this work, we propose that this type of stapling could offer a simple route to improve the biophysical properties of RHAU peptide for targeting G4 in cells.

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Figure Legends

Figure. 1. Structure analysis and design of RHAU peptide analogues. The peptide is shown in green; the guanine bases and sugar phosphate backbone of *T95-2T* in cyan and gray respectively. A) The NMR structural model of *RHAU23-T95-2T* (TTGGGTGGGTGGGTGGGT) complex (PDB No. 2N21). Residues essential for binding that make key contact with the G4 are highlighted on the *RHAU23* sequence. B) Helical wheel of RHAU specific motif with staple positions indicated.

Figure 2. A) Primary sequence of *RHAU23* (amino acid 53-70) and sequences of the non-stapled and stapled RHAU peptide variants. B) Reaction scheme for solid-phase synthesis of RHAU analogues containing a lactam bridge. Reagents and conditions: i) Pd(PPh₃)₄, DMBA, 3h; ii) Oxyma, DIC, DMF, 2h; iii) TFA, water, phenol, anisole and 1,2-ethanedithiols (33:2:2:2:1), 2h.

Figure. 3. Native gel electrophoresis binding assay between *T95-2T* and different RHAU peptides. Lanes 1 and 4 shows the migration of the free G4 *T95-2T*. G4 sample complexed with RHAU analogues containing R60E/M64K mutations are resolved in lane 2 (*RHAU LSAH₁*) and lane 3 (*RHAU AH₁*). RHAU peptides containing W65K mutation are resolved in lanes 6 (*RHAU AH₂*) and lane 7 (*RHAU LSAH₂*). The band migration of the G4 sample complexed with native *RHAU23* is shown in lane 5. N, L and S denote the native, linear and stapled version of the RHAU peptide respectively. The DNA/peptide ratio was 1:7 and the gel was revealed using UV-shadowing.

Figure. 4 The enzymatic stability of stapled RHAU against α -chymotrypsin. HPLC analysis stability of A) *RHAU AH₁* (top) and stapled analogue *RHAU LSAH₁* (bottom) and of B) *RHAU AH₂* (top) and stapled analogue *RHAU LSAH₂* (bottom) against the enzyme at timepoints 0 h, 20 h and 48 h. Elution peaks corresponding to the undigested peptide are shaded in black for clarity.

Figure. 1.

A

RHAU18	¹ HPGHLKGREIGM ¹⁰ WYAKK ¹⁸ Q
RHAU23	HPGHLKGREIGM ¹⁰ WYAKKQGGQKNK
RHAU AH ₁ (R60E/M64K)	HPGHLKG E EIG K WYAKKQGGQKNK
RHAU LSAH ₁ (R60E/M64K)	HPGHLKG E EIG K WYAKKQGGQKNK
RHAU AH ₂ (W65K)	HPGHLKGREIGM K YAKKQGGQKNK
RHAU LSAH ₂ (W65K)	HPGHLKGREIGM K YAKKQGGQKNK

Figure. 2.

Figure. 3.

Figure. 4.