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Cyclic Peptidic Mimetics of Apollo Peptides Targeting Telomeric Repeat Binding Factor 2 (TRF2) and Apollo Interaction

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ABSTRACT: Telomeric repeat binding factor 2 (TRF2) is a telomere-associated protein which plays an important role in the formation of the 3' single strand DNA overhang and the "T loop", two structures critical for the stability of the telomeres. Apollo is a 5'-exonuclease recruited by TRF2 to the telomere and contributes to the formation of the 3' single strand DNA overhang. Knocking down of Apollo can induce DNA damage response similar to that caused by the knocking down of TRF2. In this paper we report the design and synthesis of a class of cyclic peptidic mimetics of the TRFH binding motif of Apollo ($Apollo_{TBM}$). We found conformational control of the C terminal residues of $Apollo_{TBM}$ can effectively improve the binding affinity. We have obtained a crystal structure of a cyclic peptidic Apollo peptide mimetic (**34**) complexed with TRF2 which provides valuable guidance to the future design of TRF2 inhibitors.

Introduction

Shelterin is a telomere protecting complex consisting of six telomere proteins, including POT1, TPP1, TIN2, TRF1, TRF2 and Rap1. Among these six components, TRF2 plays an important role in the formation of the 3' single strand DNA overhang and the "T loop", two structures critical for the stability of the telomere[1-10]. Knocking down TRF2 can cause end-to-end joining of DNA, induce DNA damage response, and eventually leads to cell senescence and apoptosis[11-13]. Small molecules which can interact with TRF2 and modulate its functions are valuable tools in studies of shelterin and telomeres. A series of stapled peptides which can potently bind to TRF2 and block its interaction with Rap1 have been reported recently[14], but small molecule inhibitors of TRF2 with different mechanism of inhibition are still urgently needed.

TRF2 can recruit a number of accessory proteins whose functions are also critical for the protection of telomeres[15, 16]. One of these proteins is Apollo, a member of the mammalian SNM1/Pso2 family of nucleases[17, 18]. Apollo contributes to the formation of the single-stranded 3' telomere overhang, and its deletion induces DNA damage response similar to that caused by the knocking down of TRF2[19-21]. The crystal structure of an Apollo peptide (residues 496-532) in a complex with the TRFH domain of TRF2 has been determined.[10]. In this structure (**Figure 1**) the electron density of the *N*-terminal 12 residues (amino acids 498-509), the so called TRFH binding motif of Apollo ($Apollo_{TBM}$), is clearly shown. Based on this structure, Zhou et al. built a peptide library by replacing the amino acids which have been shown in the crystal structure to interact with the TRF2 protein with various natural amino acids. They found that TRF2, through its TRFH domain can selectively recognize peptides containing a [Y/F]XL sequence[22]. From this library they identified a consensus peptide which binds potently to TRF2. Expression of this peptide in tandem repeats in human cancer HTC-75 cells promotes formation of telomere dysfunction-induced foci (TIF) and induces DNA damage response, suggesting that small molecular ligands which can block the interactions between TRF2 and its associated proteins, such as Apollo, can modulate the functions of TRF2.

In common with other peptides, Apollo and its analogues have intrinsic drawbacks such as poor cell permeability and poor stability, and consequently we have tried to design peptide mimetics of $Apollo_{TBM}$ to overcome these drawbacks while maintaining or improving their binding affinity. In this paper, we report our work on the design of $Apollo_{TBM}$ mimetics as inhibitors of TRF2.

Results

To evaluate the binding affinity of our compounds to TRF2, we designed a fluorescent tracer (compound **2, Figure 2**) based on Apollo$_{TBM}$ and established a fluorescence polarization based binding assay. In the crystal structure of Apollo$_{TBM}$ complexed with the TRFH domain of TRF2 (**Figure 1**), R498 of Apollo is exposed to the solvent and has no interaction with the TRF2 protein. Consequently, in our design the R498 residue was replaced with a linker to which the fluorescence label 5-carboxy-fluorescein (5-FAM) is attached. Compound **2** has a K_d value of 179 nM, consistent with the K_d value of Apollo$_{496-532}$ in an ITC assay (K_d 120 nM)[10]. The corresponding 11-mer peptide **3** (**Table 1**) has a K_i of 380 nM in our FP based binding assay, thus proving the effectiveness of our assay.

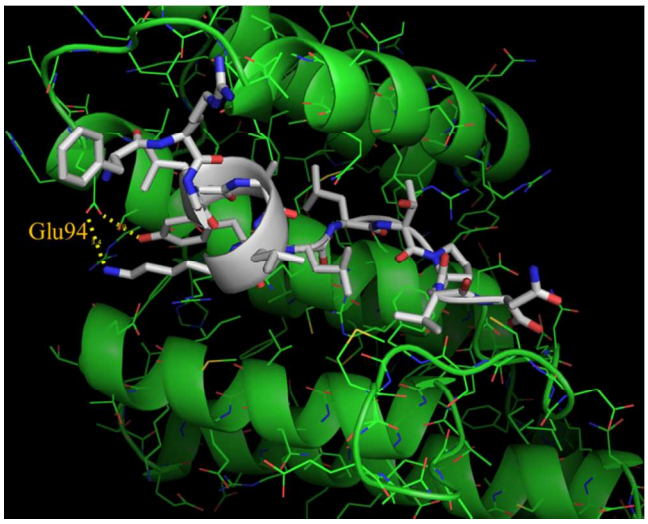


Figure 1. Crystal structure of Apollo$_{TBM}$ in a complex with the TRFH domain of TRF2 (PDB 3bua)

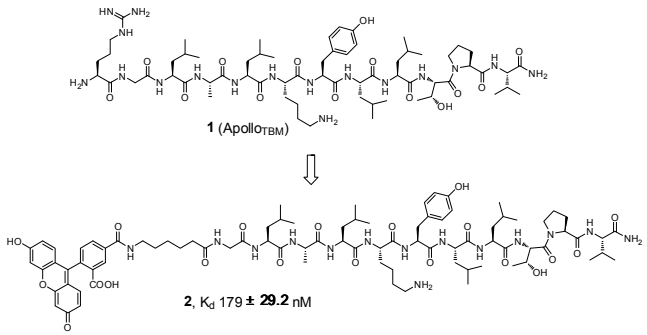


Figure 2. Design of the fluorescent tracer for TRF2

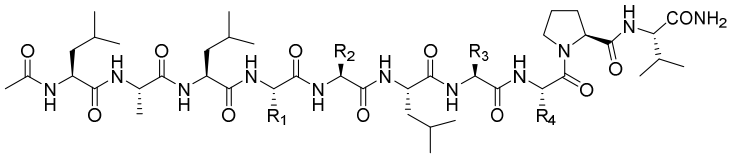
We then performed truncation studies at both the *N*-terminal and *C*-terminal regions of the 11-mer peptide (**3**) with the purpose of identifying the shortest peptide sequence which can maintain a reasonable binding affinity to Apollo, and the results are summarized in **Table 1**. Of the *N*-terminal truncations, removal of the G499 residue decreases the binding by about 7 fold (**4** vs **3**). In the crystal structure this residue has no interaction with the protein, but its carbonyl group forms an intramolecular hydrogen bond with the α-amino group of K503 of Apollo. To assess the importance of this hydrogen bond, we designed compound **5** in which the G499 residue in **3** is replaced by an acetyl group. With a K_i of 0.34 μM, compound **5** is as potent as **3**, indicating that the conformation controlled by this hydrogen bond is critical to the binding. The side chain of L500 in **5** binds to a large but shallow hydrophobic pocket in the TRF2 protein, and removal of this residue decreases the binding by about 7 fold (**6** vs **4**). In the crystal structure the methyl group of A501 can be seen to occupy a small hydrophobic pocket in the TRF2 protein, while the side chain of the neighboring residue, L502 is exposed to the solvent and has no interaction with the protein. Our truncation studies however indicate that removal of A501 doesn't influence the binding affinity (**7** vs **6**), but removal of L502 decreases the binding by about 5 fold (**8** vs **7**). These results suggest that these truncated peptides could bind to the TRF2 protein with a conformation different from that of the original peptide **3**. Compound **8** (KYLLTPV) binds only weakly to TRF2 with a K_i of 122 μM, so further truncation of this end of the peptide was not pursued.

Table 1. Truncation studies for Apollo peptide

Compound Number	Peptides	Ki (μM)
3	GLALKYLLTPV	0.38±0.12
4	LALKYLLTPV	2.2±0.34
5	AcLALKYLLTPV	0.34±0.08
6	ALKYLLTPV	22.8±2.6
7	LKYLLTPV	26.8±3.2
8	KYLLTPV	122.6±8.4
9	AcLALKYLLTP	1.89±0.23
10	AcLALKYLLT	26.3±2.7
11	AcLALKYLL	98.0±9.6
12	AcLALKYL	461±34

C-terminal truncations were started from compound **5** which is as potent as **3**. The crystal structure of Apollo$_{TBM}$ complexed with TRF2 indicates that the C-terminal residues of the peptide have multiple hydrophobic interactions with the protein. The side chain of V509 has a hydrophobic interaction with M122 in TRF2, and we found that removal of this residue decreases the binding affinity by a factor of about 5 fold (**9** vs **5**). The pyrolidine ring of P508 has a hydrophobic interaction with Phe120 of TRF2 and Lei et al. found that mutation of Phe120 to alanine completely abolishes the binding of TRF2 to Apollo, indicating that this hydrophobic interaction is critical. We found that, consistent with the previous mutation study, removal of P508 reduces the binding affinity by about 14 fold (**10** vs **9**). Notwithstanding the apparent absence of interaction between T507 and the Apollo protein, removal of T507 causes about a 4 fold decrease in binding affinity (**11** vs **10**), possibly because of conformational changes. The side chain of L506 binds to a deep hydrophobic pocket in TRF2 and mutation studies performed by Lei et al. have confirmed that this is the most important interaction in the binding of the Apollo protein to TRF2. Our own truncation study supports this in that after removal of L506 the resulting 5-mer peptide (compound **12**) retains only a very weak binding affinity to TRF2 with a K_i of 461 μM. Overall, our truncation studies confirm the SAR information obtained from the Apollo$_{TBM}$-TRF2 crystal structure, and indicate that among all the truncated peptides, only the acetylated 10-mer peptide (**5**) maintains the good binding affinity. Consequently, this peptide was used as the lead compound in further modifications.

Table 2. Structure-activity relationship studies for compound **5.**



Peptides	R_1	R_2	R_3	R_4	Binding affinity to TRF2 (K_i, μM)
13	methyl				3.2±0.40
14					3.4±0.52
15					0.72±0.13
16			methyl		>100
17			propyl		4.7±0.34
18			isopropyl		61.2±5.80
19					11.3±1.80
20					6.1±0.77
21			cyclopentyl		0.51±0.08
22			cyclohexyl		0.21±0.09
23					1.4±0.21
24					8.5±0.67
25				methyl	0.24±0.08

To gain additional structure-activity relationships (SAR) for this class of peptides, we performed mutation studies for several residues which have been shown to be critical to the binding affinity and the results are reported in **Table 2**. The $Apollo_{TBM}$-TRF2 crystal structure indicates that the ε-amino group of K503 and the hydroxyl group of Y504 of Apollo have electrostatic interactions with Glu94 of TRF2 (**Figure 1**). In order to explore the significance of these interactions, we designed three mutated peptides **13-15**. In **13**, K503 was replaced with an alanine, while in **14** and **15**, the side chain of Y504 was replaced with benzyl or a 4-aminobenzyl, respectively. **13** and **14** are about 10 times less potent than **5** with K_i values of 3.2 and 3.4 μM respectively, suggesting that the electrostatic interactions from both K503 and Y504 are important to the binding affinity. **15**, with a K_i of 0.72 μM, is only 2 times less potent than **5** and about 5 times more potent than **14**, confirming the importance of the electrostatic interaction between **15** and the Glu94 residue in the protein. Because the hydrophobic interaction of the side chain of L506 with a deep hydrophobic pocket in TRF2 has been shown to be the most critical to the binding affinity, we performed extensive modifications to this residue, replacing it with a series of natural and unnatural amino acids containing different hydrophobic groups. Among all the amino acids tested, the binding affinity is maintained or slightly improved only when L506 is replaced with cyclopentyl glycine (**21**) or cyclohexyl glycine (**22**). All other modifications at this site result in a decrease in the binding affinity. T507 has no interaction with the protein, but the hydroxyl group in this threonine residue can cause some synthetic inconvenience due to the need for protection and deprotection. Accordingly, we replaced this residue with an alanine and found the resulting compound (**25**) is as potent as **5**, therefore subsequently, **25** was used as the new lead compound for further modifications.

Figure 3. Design of cyclic Apollo peptide mimetics

From the crystal structure, we observed that the six N-terminal residues GLALKY in **3** are part of an α-helix, while the five *C*-terminal residues LLTPV form a flexible loop (**Figure 1**). In α-helix, the peptide bonds are all involved in intramolecular hydrogen bonds, with only the side chains participating in the interactions with the proteins, so for *N*-terminal residues we planned to replace the helical peptide with a non-peptide scaffold to which different substituent groups could be attached to mimic the side chains of the residues which interact with the protein. For the *C*-terminal residues, the conformational flexibility is a disadvantage for modification because it can compromise the SAR, and thus we sought to constrain the conformation flexibility of these residues. In the crystal structure the side chains of L505 and V509 are very close to one another (**Figure 1**). Therefore, we proposed that these two residues could be linked to form a conformationally constrained cyclic peptide.

To test this strategy, we first designed and synthesized a cyclic peptide **27** (**Figure 3**) by replacing the side chains of

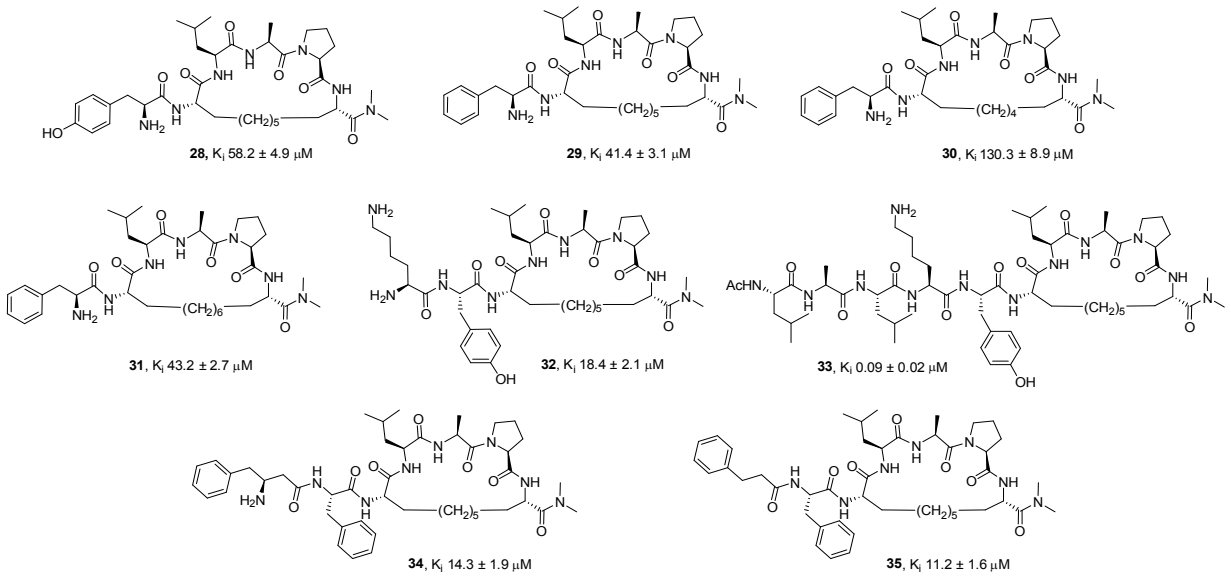


Figure 4. Structures and activities of cyclic peptide mimetics of Apollo peptides

L505 and V509 in the *C*-terminal 5-mer Apollo peptide (**26**) with a linker containing seven methylene groups corresponding to the distance between these two side chains in the crystal structure. In our FP based binding assay, compound **27** with a K_i of 115 μM, is at least three times more potent than the 5-mer peptide **26** (K_i > 500 μM).

Encouraged with the improved binding affinity for **27** over **26**, we then designed compounds **28** and **29** by introducing a Tyr or Phe to the amino group of **27** (**Figure 4**). Compounds **28** and **29** were found to have comparable binding affinity to TRF2 with K_i values of 58 and 41 μM respectively, and are 3 to 4 times more potent than **27**, suggesting that the hydroxyl group in **28** may fail to interact with Glu94 in the TRF2 protein. We have explored the influence of the length of linker to the binding affinity of the cyclic peptides by designing compounds **30** and **31** (**Figure 3**) by replacing the 7 methylene linker in **29** with a spacer containing 6 or 8 methylene groups, respectively. The peptide **30** is 3 times less potent than **29** but **31** is as potent as **29**, indicating that linkers with 7 or 8 methylene groups are more optimal for cyclic peptides. In order to confirm that a cyclic peptide can be used to replace the C-terminal linker peptide, we designed the 7-mer analogue **32** and the 10-mer analogue **33** by re-introduction of the appropriate $Apollo_{TBM}$ *N*-terminal residues to **28**. Compound **32** binds to TRF2 with a K_i of 18 μM, and is 7 times more potent than the 7-mer peptide **8**, while **33** binds to TRF2 with a K_i of 90 nM, and is 3 times more potent than the 10-mer peptide **5**, proving that conformational control of the *C*-terminal residues can effectively improve the binding affinity.

We also examined *N*-terminal modifications of **29**. Compound **34**, in which a (*S*)-3-amino-4-phenylbutyric acid was introduced to the free amino group of **29**, has a K_i of 14.7 μM, and is about 3 times more potent than **29**. The synthetic cyclic peptidic mimetics have been screened by co-crystallization with the TRF2 TRFH domain protein. The complex of **34** with TRF2 gave a crystal structure with a resolution of 2.05 Å (**Figure 5**). In this crystal structure, **34** can be seen to bind to the same area in the TRFH domain of TRF2 as the *C*-terminal five residues of $Apollo_{TBM}$. The conformation of the protein is very close to that of $Apollo_{TBM}$ with TRF2, with the exception of the helix containing Glu94. When binding to the wild type peptide, this helix is distorted because of the interactions of Glu94 with the ε-amino group in K503 and the OH group in Y504 (**Figure 1**). But when binding to **34**, Glu94 flips back and has no interaction with the compound, suggesting that the interaction between Glu94 and the amino group in **34** is not sufficient to cause the distortion of the helix. This also explains why the Tyr-containing compound **28** is as potent as the Phe-containing compound **29**. The amino group of (*S*)-3-amino-4-phenylbutyric acid is exposed to the solvent, while its phenyl group binds to a large hydrophobic area where the N-terminal residues bind.

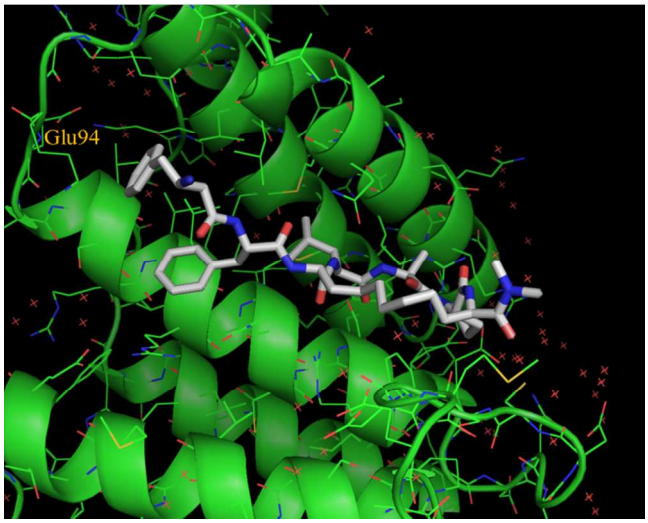


Figure 5. Crystal structure of compound 34 in a complex with TRF2

Based on this crystal structure, we designed compound **35** (**Figure 4**) by introduction of a 3-phenylpropanoic acid to the amino group of **29**. This compound has a Ki of 11.2 μM, indi-

cating that introduction of a hydrophobic group to the *N*-terminal indeed improves the binding affinity. This crystal structure thus provides the foundation for our future design.

Summary

We have designed a fluorescent tracer based on the crystal structure of $Apollo_{TBM}$ in a complex with the TRFH domain of TRF2 and developed a competitive binding assay based on fluorescence-polarization for this domain of TRF2. Through truncation and mutation studies we identified an acetylated 10-mer peptide (**25**) which can bind to TRF2 with a binding affinity comparable to that of $Apollo_{TBM}$. Based on the *C*-terminal five residues in **25**, we designed and synthesized a series of cyclopeptidic mimetics and found that replacement of the five *C*-terminal residues in **25** with our designed cyclic peptide can effectively improve the binding affinity. Our study has yielded a cyclic peptide **33**, which binds to TRF2 with a K_i value of 90 nM. We solved the crystal structure of one of our cyclic peptide analogues (**34**) in a complex with TRF2, and found that this compound binds to the same site in TRF2 as the original $Apollo_{TBM}$ peptide. Further optimizations to these cyclic peptides are in progress and the results will be reported subsequently.

ASSOCIATED CONTENT

Supporting Information

The procedure and experimental detail for solid phase synthesis of the truncated and mutated peptides, chemical synthesis of the cyclic peptidic mimetics, saturation binding experiment to determine dissociation constant (K_d) of the tracer, competitive binding experiment to determine the binding affinities of the synthesized compounds, crystallization of the complex of **34** with TRF2 and structural determination of the crystal

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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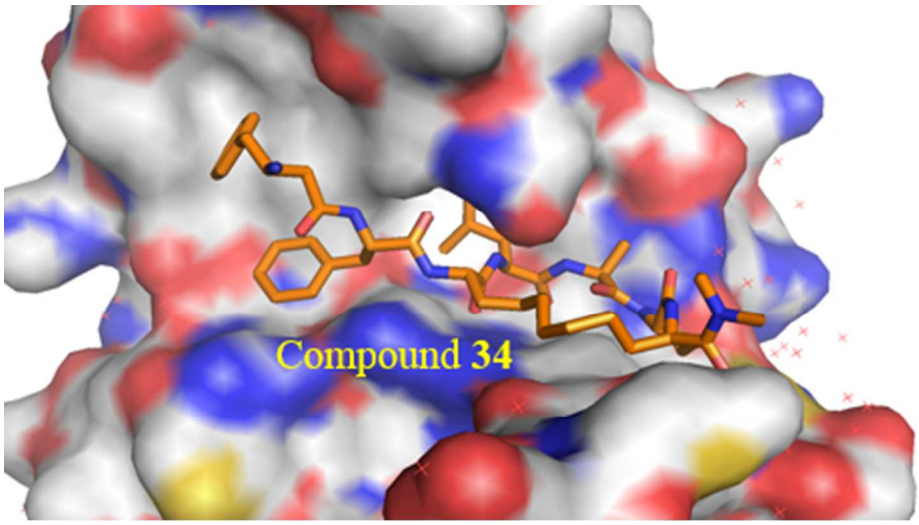

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