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Peptoid-Peptide Hybrid Ligands Targeting the Polo Box Domain of Polo-Like Kinase 1

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Abstract

We replaced the amino terminal Pro residue of the Plk1 polo box domain-binding 5-mer peptide (PLHSpT) with a library of *N*-alkyl-Gly “peptoids” and identified long-chain tethered phenyl moieties giving greater than two-orders-of-magnitude affinity enhancement. Further simplification by replacing the peptoid residue with appropriate amides gave low-nanomolar affinity *N*-acylated tetrapeptides. Binding of the *N*-terminal long-chain phenyl extension was made clear by X-ray co-crystal data.

Keywords

kinases; molecular recognition; proteins; crystallography

Introduction

The polo-like serine/threonine protein kinases (Plks) are important mediators of cell cycle regulation that contain C-terminal polo-box domains (PBDs) whose function is to bind to phosphoserine (pS)/phosphothreonine (pT)-containing sequences. Because Plk1 can promote oncogenic transformation, efforts are underway to develop PBD-binding inhibitors as potential down-regulators of Plk1 function.^[1] We have taken the crystal structure of the 5-mer PLHSpT peptide (**1**) bound to the Plk1 PBD (PDB: 3HIK) as a starting point for antagonist development.^[2] The structure shows that the *N*-terminal Pro residue interacts with the PBD surface formed by the two aromatic residues Trp414 and Phe535 and by

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hydrogen bonding of its carbonyl group with the guanidinium moiety of Arg516. This orientation differs greatly from that of the *N*-terminal Pro residue of PBD-bound PPHSpT (PDB: 3C5L), such that the combined protein-binding surface encompassed by these two orientations covers a broad region. Therefore, we speculated that the *N*-terminal Pro residue of **1** could potentially provide attractive opportunities for structural elaboration. Proline is distinguished among the encoded amino acids by having a secondary α -amino group that is derived from intramolecular alkylation to form a pyrrolidine ring. *N*-substituted glycines (NSGs) are a class of secondary amine-containing constructs that present features reminiscent of Pro residues.^[3] Pseudopeptides composed of NSGs (termed “peptoids”^[4]) have shown utility in drug discovery,^[5] as have peptide–peptoid hybrids, which contain both peptide and NSG residues.^[6] Our current study was undertaken to optimize the *N*-terminal PBD binding interactions of peptide **1** by using a peptide-peptoid hybrid approach that employed NSG residues in place of the Pro residue at the pT-4 position.

Results and Discussion

We synthesized a library of peptide-peptoid hybrids using acid sensitive NovaSyn® TGR resin using Fmoc protocols and a “submonomer approach.”^[7] Resin-bound peptide **2** was *N*-terminally deprotected to provide a free Leu residue (representing the -3 position of parent peptide **1**), which was bromoacetylated to yield the corresponding α -bromoacetamide **3** (Scheme 1).

This peptide served as the common intermediate for subsequent analogs. The resin was divided into portions and each portion was treated with an appropriate amine (**4a** – **4r**, Scheme 1) to yield the corresponding NSG-containing derivative (**5a** – **5r**). Acetylation of the terminal secondary amines gave the fully protected resin-bound congeners, which were cleaved under acidic conditions to provide the final peptoid-peptide hybrids as their *C*-terminal carboxamides (**6a** – **6r**, Scheme 1).

The Plk1 PBD binding affinities of the synthetic products were evaluated using an ELISA-based 96-well assay that measures their ability to compete with a surface bound high affinity pT-containing peptide for binding to Plk1 in mitotic 293A cell lysates.^[2] Hybrids **6d** and **6e** had dramatically diminished binding affinity relative to the parent peptide. This indicated that both positive and negative charges are not well tolerated in this region of the molecule. The hybrids **6f**, **6j** and **6k** showed affinities similar to the parent peptide **1**, while **6a**, **6c**, **6g** and **6i** showed slightly higher binding affinities than **1** and the analogues **6b**, **6h** and **6l** were weaker binders than **1** (Supporting Information Figure S1). The binding affinities of **6a** – **6l** indicated that non-charged, hydrophobic groups of limited bulk were preferred.

Keying on analogues **6f** – **6j**, we explored phenyl functionality tethered by a series of (CH₂)*n*-based linkers having sequentially increasing chain lengths from *n* = 2 to *n* = 7 (**6m** – **6r**, Scheme 1). The binding affinities of these peptoid-peptide hybrids increased with increasing linker length, reaching a maximum at *n* = 6 (**6q**), with *n* = 7 (**6r**) being of equal potency (Figure 1a). Since *n*-alkylphenyl groups impart considerable hydrophobic character, we were concerned that these results may reflect ‘promiscuous’ binding mechanisms that do not rely on specific interactions with the PBD.^[8] To examine this possibility, we made use

of the fact that the ‘SpT’ dipeptide motif is crucial for specific high-affinity Plk1 PBD binding and that substitution of the serine residue by an alanine markedly reduces affinity.^[9] We replaced the serine residue in **6q** with an alanine residue [**6q(S4A)**] and found that this resulted in a significant reduction in binding affinity (Figure 1A). We concluded from this that binding of **6q** was specific.

Our previous work has shown that the tetrapeptide “LHSpT”, obtained by deletion of the *N*-terminal Pro (-4 residue), exhibits significantly less Plk1 PBD-binding affinity than the parent peptide **1**.^[10] However, the binding affinity of **6q** appeared to derive significantly from the tethered phenyl functionality. Therefore, we prepared the tetrapeptide **7** by acylating the resin-bound **2** with an *N*-terminal Phe-(CH₂)₈-CO-group prior to cleavage from the resin (Scheme 2). The total extension of the tethered phenyl moiety in **7** is approximately the same as the alkylated NSG residue in **6q**. The Plk1 PBD-binding affinity of **7** equaled that of the pentapeptide **6q**. This indicated that the *N*-terminal portions of both peptides may bind in a similar fashion (Figure 1B).

Because the tether chains in both **6q** and **7** are highly flexible, it was unclear what the effects would be of introducing conformational restriction in this region of the peptide. Copper(I)-catalyzed [3+2] Huisgen cycloaddition “click chemistry” of azides and alkynes is a widely used means of functional group ligation that yields substituted triazole rings having fixed geometries.^[11] Accordingly, we converted resin-bound **2** to its *N*-terminal pent-4-ynamide (**8**) and subjected this to copper(I)-catalyzed Huisgen cycloaddition using azides **9** (three carbon chain) and **10** (four carbon chain) (Scheme 2). When the reactions were performed in DMSO at room temperature overnight the 1,4-substituted triazole products (**11a** and **12a**) were obtained. However, when the reactions were run in DMF at 100 °C for 48 h without copper, the sterically more crowded 1,5-substituted products (**11b** and **12b**) were also obtained along with the 1,4-substituted products (Scheme 2). The binding affinities of all four triazole-containing peptides were reduced relative to **6q** and **7** (Figure 1B). This potentially indicates unfavorable conformationally-induced interactions. The reduced affinities of **11a** and **12a** are noteworthy, since their 1,4-substituted triazoles closely mimic the NSG residues in peptoid-peptide hybrids **6p** and **6q**, respectively.

In order to compliment ELISA binding data obtained on solid support, we performed Plk1 PBD competition assays using fluorescence polarization techniques as previously described.^[12] This assay measures the ability of unlabeled peptides to compete with 5-carboxyfluorescein-GPMQSpTPLNG-OH for binding to PBD protein in solution. It was found (Supporting Information Figure S2) that the parent peptide **1** showed 40 ± 0.2% inhibition at the highest concentration tested (2.56 μM). In contrast, the peptoid-peptide hybrid **6q**, which along with **6r**, was identified by ELISA assays as being the most potent analogue tested, exhibited greater than 170-fold higher affinity (IC₅₀ = 15 ± 0.3 nM) (Table 1). Also consistent with the ELISA data in Figure 1B was the finding that the tetrapeptide **7** (IC₅₀ = 19 ± 0.3 nM) was only slightly less potent than **6q**. We determined the effects of replacing the pThr residue with the phosphatase-stable pThr mimetic (2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutyric acid (Pmab).^[13] Peptides containing Pmab could be potentially advantageous over their pThr-containing counterparts in cellular studies, where cytoplasmic

phosphatases can cleave phosphoryl ester bonds.^[2] When the pThr residue in **6q** was replaced with Pmab to give the corresponding peptide **13** (Scheme 3) a moderate reduction in affinity was observed (**13**, $IC_{50} = 71 \pm 17$ nM). However, binding was still specific, since the mutant **13(S4A)** variant resulted in a nearly 500-fold loss of affinity (Table 1).

A fluorescein isothiocyanate (FITC)-labeled PEGylated version of **6q** was prepared (**14**, Scheme 3) for use in fluorescence polarization-based determination of binding affinities to the PBDs of Plk1, 2 and 3.^[12] For each domain, K_d -values were extrapolated from the fluorescence polarization binding curves as the concentration at which 50% of the fluorescein-labeled peptide **14** is protein-bound (Supporting Information Figure S3). Against the Plk1 PBD, a K_d value of 26.7 ± 2.8 nM was obtained, which is consistent with the IC_{50} value of 15 nM found in fluorescence polarization competition assays (see above). In contrast, K_d values against the PBDs of Plk2 and Plk3 were more than two-orders of magnitude higher (> 2560 nM) (Supporting Information Figure S3).

In order to determine the molecular basis for the enhanced binding affinity of **6q** relative to the parent peptide **1**, we solved the X-ray co-crystal structure of **6q** in complex with Plk1 PBD (Supporting Information Table S4 and Figure S4). The residues “HSpT” of **6q** were nearly super-imposable with those of the PBD-bound **1** in the 3HIK structure (Figure 3). However, significant structural differences were observed with the pT-3 Leu residue, where the psi angles ($\psi = -1.9^\circ$ and -141.5° for **1** and **6q**, respectively) place the *N*-terminal functionality of these peptides in nearly opposing directions. In this new orientation, the peptoid Phe(CH₂)₆- group of **6q** is directed across the $\beta 2$ and $\beta 3$ sheets of the PBD protein, where it terminates with its phenyl ring nestled against the αB helix. The net effect is to reveal a channel that is absent in the binding of the parent peptide **1**. Revelation of this binding channel is made possible by extensive downward rotation of the Y481 side chain, which occludes access in the 3HIK structure. Other residues lining the new channel (V415, Y421, F482 and Y485) have orientations only modestly different from those observed in the 3HIK structure (Figure 2).

Interactions of the peptoid Phe(CH₂)₆- group of **6q** are nearly superimposable with what is seen when 4*R*-Phe(CH₂)₄-O- functionality is introduced at the amino-terminal Pro residue of the parent peptide **1** (PBD: 4DFW) (Figure 3A).^[2b] We observed similar interactions with a Phe(CH₂)₈ adduct originating from the histidine imidazole ring of **1** (PDB: 3RQ7) (Figure 3B).^[2a] Additionally, the binding of the phenyl ring of **6q** is also related to what has been reported for the phenyl ring in the *N*-terminal Phe residue of “FDPLHSpTA” (PDB: 3P37) (Figure 3C).^[14] It can also be surmised for tetrapeptide **7** that the *N*-terminal Phe(CH₂)₈CO- group binds in a fashion similar to **6q**. Taken together, these findings indicate a high degree of plasticity in the protein that permits diverse structures to access the auxiliary pocket.

Conclusion

The original intent of this study was to optimize *N*-terminal PBD-binding interactions of the WT peptide **1** by replacing its Pro residue with NSG constructs. These efforts were highly successful. They yielded greater than two orders-of-magnitude improved affinity over the parent peptide while maintaining good Plk1 PBD selectivity. We achieved further

simplification by replacing the peptoid altogether. Our findings expand the design parameters available for development of Plk1 PBD-binding antagonists by showing that low-nanomolar affinity tetrapeptide analogues can be derived by appropriate *N*-terminal acylation. These results should be useful for further development of high affinity PBD-binding peptides and peptide mimetics.

Experimental Section

General Synthetic Procedures

All experiments involving moisture-sensitive compounds were conducted under dry conditions (positive argon pressure) using standard syringe, cannula, and septa apparatus. Solvents: All solvents were purchased anhydrous (Aldrich) and used directly. HPLC-grade hexanes, EtOAc, CH₂Cl₂, and MeOH were used in chromatography. TLC: analytical TLC was performed on Analtech pre-coated plates (Uniplate, silica gel GHF, 250 microns) containing a fluorescence indicator; NMR spectra were recorded using a Varian Inova 400 MHz spectrometer. Coupling constants are reported in Hertz, and peak shifts are reported in δ (ppm) relative to TMS. Low resolution mass spectra (ESI) was measured with Agilent 1200 LC/MSD-SL system, and high resolution mass spectra (ESI or APCI) were obtained from the UCR Mass Spectrometry Facility, Department of Chemistry, University of California, 3401 Watkins Dr., Riverside CA, 92521.

Analytical HPLC Analysis

The purities were determined by using Phenomenex C₁₈ column (4.60 mm dia $\times$ 250 mm, cat. no: 00G-4435-E0) with a linear gradient from 5% aqueous acetonitrile (0.1% trifluoroacetic acid) to 100% acetonitrile (0.1% trifluoroacetic acid) over 25 minutes at a flow rate of 1.0 mL/minute, followed by 100% acetonitrile for another 5 minutes.

Synthesis of Peptoid-peptide Hybrids (6a – 6r)

Fmoc-Thr(PO(OBzl)OH)-OH and other Fmoc-protected amino acids were purchased from Novabiochem. Peptides were synthesized on NovaSyn[®]TGR resin (Novabiochem, cat. no. 01-64-0060) using standard Fmoc solid-phase protocols in *N*-methyl-2-pyrrolidone (NMP). 1-*O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (5.0 equivalents), hydroxybenzotriazole (HOBt) (5.0 equivalents) and *N,N*-diisopropylethylamine (DIPEA) (10.0 equivalents) were used as coupling reagents. Coupling of the Leu residue and Fmoc-deprotection provided the resin-bound peptide **2** having a free terminal amine group. Treatment with bromoacetic acid (10.0 equivalents), DIC (5.0 equivalents) and DIPEA (20.0 equivalents) in NMP (30 minutes) followed by washing provided the corresponding resin-bound bromoacetylated peptide (**3**). Aliquots of the resin were treated with primary **4a – 4r** in DMSO ($\approx$ 2.5 M, overnight) to provide the corresponding resin-bound peptides **5a – 5r** (Scheme 2). Amines **4a – 4o** were purchased from Sigma Aldrich, while amines **4p – 4r** were prepared according to the literature procedures.^[15] Terminal alkylpeptide amines were acetylated using either acetylimidazole (5.0 equivalents in DMF, overnight for peptides **5a – 5l**), or acetic anhydride (10.0 equivalents) in the presence of DIPEA (20.0 equivalents) in 1,2-dichloroethane (room temperature, 4 h for peptides **5m – 5r**). Incorporation of (2*S*,3*R*)-2-amino-3-methyl-4-

phosphonobutyric acid (Pmab) in place of the pThr residue was accomplished as previously reported.^[13] For peptide pull-down assays, a cysteine residue was attached to the amino-terminus by means of a 6-aminohexanoylamide (Ahex) spacer. Completed resins were sequentially washed with DMF, MeOH, CH₂Cl₂ and ether and then dried under vacuum (overnight). Resins (200 mg) were cleaved by treatment with a mixture of trifluoroacetic acid : triisopropylsilane : H₂O (90 : 5 : 5) (5 mL, 4 h) and concentrated under vacuum and then diluted with cold ether to give precipitated products, which were washed with ether. The resulting solids were dissolved in 50% aqueous acetonitrile (5 mL) and purified by reverse phase preparative HPLC using a Phenomenex C₁₈ column (21 mm dia × 250 mm, cat. no: 00G-4436-P0) with a linear gradient from 0% aqueous acetonitrile (0.1% trifluoroacetic acid) to 100% acetonitrile (0.1% trifluoroacetic acid) over 30 minutes at a flow rate of 10.0 mL/minute. Lyophilization gave product peptides **6a** – **6r** as white powders. Mass spectral and HPLC data are provided in the Supporting Information (Tables S1 – S3).

On-resin Modification of Peptides by Click Chemistry: Synthesis of Peptides **12** and **13**

Resin-bound peptide **2** (0.20 mmol) was converted to the corresponding amino-terminal 4-pentynylamide (**8**) by reaction with 4-pentynoic acid (5.0 equivalents), DIC (5.0 equivalents), HOBT (5.0 equivalents) and DIPEA (20.0 equivalents) in NMP (3.0 mL) at room temperature (2 h). The resin was washed with DMF, CH₂Cl₂, MeOH and ether and dried under high vacuum (overnight). Conversion of 3-phenylpropanol and 4-phenylbutanol to the corresponding 3- and 4-azido derivatives **9** and **10** respectively, was accomplished by treatment with methanesulfonyl chloride followed by sodium azide. Copper catalyzed azide – alkyne cycloaddition reactions were conducted both with and without warming to provide the corresponding 1,4-triazole adducts (**11a** and **12a**) and 1,5- triazole adducts (**11b** and **12b**), respectively. For reactions performed without heat, dried resin (**8**, 100 mg) was suspended in acetonitrile (4.0 mL) with DMSO (1.0 mL) in a plastic tube and de-gassed under argon (5 minutes). To this was added DIPEA (10 µL), CuI (19 mg) and phenyl alkyl azide (**9** or **10**, 10.0 equivalents) and the tube was sealed and agitated at room temperature (overnight). The resin was washed sequentially with DMF, H₂O, MeOH and ether and dried under high vacuum (4 h). Cleavage as described above provided 1,4-triazole adducts (**11a** and **12a**) following HPLC purification. For reactions performed with heating, dried resin (**8**, 100 mg) and phenyl alkyl azide (**9** or **10**, 10.0 equivalents) were mixed in DMF (2.0 mL) in a flask and heated (100 °C, 2 days). The resins were then washed sequentially with DMF, H₂O, MeOH and ether and then dried under high vacuum (4 h). Cleavage as described above provided 1,5- triazole adducts (**11b** and **12b**), respectively following HPLC purification. Mass spectral and HPLC data are provided in the Supporting Information (Table S2).

ELISA-based PBD-binding Inhibition Assays

Peptide pull-down assays were carried out essentially as described previously.^[2, 10] A biotinylated p-T78 peptide was first diluted with 1X coating solution (KPL Inc., Gaithersburg, MD) to a final concentration of 0.3 µM, and then an aliquote of the resulting solution (100 µL) was immobilized onto a 96-well streptavidin-coated plate (Nalgene Nunc, Rochester, NY). The wells were washed once with PBS plus 0.05% Tween 20 (PBST), and

incubated with PBS (200 μ L) plus 1% BSA (blocking buffer) (1 h) to prevent non-specific binding. Mitotic 293A lysates expressing HA-EGFP-Plk1 were prepared in TBSN buffer (~60 μ g total lysates in 100 μ L buffer), mixed with the indicated amount of peptide ligands and applied immediately onto the biotinylated p-T78 peptide-coated ELISA wells, and then incubated with constant rocking (1 h at 25 $^{\circ}$ C). Following incubation, the ELISA plates were washed with PBST (4 times). To detect bound HA-EGFP-Plk1, the plates were probed (2 h) with anti-HA antibody (100 μ L/well, 0.5 μ g/mL in blocking buffer) and then washed (5 times). The plates were further probed (1 h) with HRP-conjugated secondary antibody (100 μ L/well, a 1:1,000 dilution in blocking buffer) (GE Healthcare, Piscataway, NJ). The plates were washed with PBST (5 times) and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (100 μ L/well) (Sigma, St. Louis, MO) until a desired absorbance was achieved. The reactions were stopped by the addition of stop solution (100 μ L/well) (Cell Signalling Technology, Danvers, MA) and the optical densities (O.D.) were measured at 450 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). Data are shown in Figure 1 and Supporting Information Figure S1.

Plk1 PBD fluorescence polarization competition binding assays

Competition assays for the Plk1 PBD were performed essentially as described.^[12] In brief, 5-carboxyfluorescein-GPMQSpTPLNG-OH (final concentration: 2 nM) was incubated with the Plk1 PBD (final concentration: 20 nM) in the presence of the test peptides (final concentrations of buffer components: 50 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.1% Nonidet P-40 substitute, 1 mM dithiothreitol, and 2% DMSO.) Fluorescence polarization was analyzed after 60 minutes. Inhibition curves were fitted using SigmaPlot (SPSS) and shown in Supporting Information Figure S2. All experiments were performed in triplicate. Numerical IC₅₀ values are shown in Table 1.

X-ray crystallography

Procedures, refinement statistics (Table S4) and sigmaA weighted 2Fo-Fc electron density map contoured at 1 σ and 1.55 $\text{\AA}$ resolution around the ligand **6q** (Figure S4) are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
ELISA-based Plk1 PBD-binding results. Representative graphs from three independent experiments are shown (O.D. equals optical density).

Figure 2. Comparison of Plk1 PBD in complex with peptide **1** (carbons in magenta) superimposed with bound **6q** (carbons in grey). For **6q**, protein side chains lining the Phe(CH₂)₆-binding channel are shown in yellow with the Y481 side chain associated with bound **1** shown in magenta.

Figure 3. Superposition of Plk1 PBD-bound 6q (carbons in grey) over previously reported PBD-bound peptides. PBD codes for bound peptides: A) 4DFW; B) 3RQ7; C) 3P37.

Scheme 1.
Submonomer solid-phase synthesis of **6a** – **6r**.

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Scheme 3.
Variants of peptide **6q**.

Table 1Plk1 PBF-binding IC₅₀ values.^[a]

No	IC ₅₀ [μM]
1	[b, c]
6q	0.015 ± 0.003
7	0.019 ± 0.002
13	0.071 ± 0.017
13(S4A)	34.02 ± 2.88

^[a] Determined by competition against binding of 5-carboxyfluorescein-GPMQSpTPLNG-OH (5-CF-15) and the Plk1 PBD as determined by fluorescence polarization assays.

^[b] 40 ± 2% inhibition at 2.56 μM, (reference 2a).

^[c] Autofluorescence-limited.