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Structure-guided design of a potent peptide inhibitor targeting the interaction between CRK and ABL kinase†

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CT-10 regulator of kinase (CRK) proteins play important roles in human cancer metastasis and invasion. Moreover, CRK proteins are the major phosphorylation substrates of ABL kinase and its oncogenic mutant BCR-ABL kinase. The interaction between CRK and BCR-ABL plays important roles in chronic myeloid leukemia. Hence, inhibiting the interaction of CRK with BCR-ABL is an attractive way to attenuate cancer metastasis. Herein, we report the development of a peptide inhibitor, PRM-3, targeting the interaction between CRK-II and ABL kinase. PRM-3 binds to the N-terminal SH3 (nSH3) domain in CRK-II with a 10 nM affinity and prevents the interaction between CRK-II and ABL kinase. An *in vitro* biochemical assay demonstrated that PRM-3 inhibits the ABL-dependent phosphorylation of CRK-II more effectively than imatinib. Remarkably, PRM-3 also inhibited the CRK phosphorylation by T315I-ABL kinase, which is resistant to all first- and second-generation tyrosine kinase inhibitors. Our study provides a promising alternative approach to overcome the drug resistance of ABL kinase.

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Introduction

Inhibiting protein–protein interactions (PPIs) has emerged as an attractive strategy for overcoming drug resistance.^{1,2} However, binding interfaces of protein complexes are often suboptimal for the development of high-affinity inhibitors, because they are shallow and lack deep hydrophobic pockets. One such example is the Src homology 3 (SH3) domain.³ While it plays critical roles as the “molecular Velcro” in numerous cellular signaling pathways,⁴ the SH3 domain has proved to be a difficult target for drug development because of its weak affinity to cognate proline-rich motifs (PRMs), with K_d ranging from 1 to 100 μM .⁵ Despite recent advances,^{6,7} the development of high-affinity SH3 ligands remains a challenge.

The CT-10 regulator of kinase (CRK) family proteins (CRK-I, CRK-II, and CRK-L) play important roles in cell proliferation and are involved in diverse human cancers.^{8–10} CRK proteins are the major phosphorylation substrates of ABL kinase and its oncogenic form BCR-ABL,^{11–13} which is found in ~90% of patients with chronic myelogenous leukemia (CML).¹⁴ The interaction of CRK proteins with ABL/BCR-ABL kinase (CRK:ABL interaction) is mediated by the binding be-

tween the N-terminal SH3 (nSH3^{CRK}) domain of CRK and proline-rich motifs (PRMs) in ABL (Scheme 1).^{15,16} Upon nSH3^{CRK}:PRM^{ABL} binding, the ABL kinase domain phosphorylates CRK-II,¹⁷ which in turn transactivates ABL kinase activity.¹⁸ Hence, the phosphorylation level of CRK proteins is considered to be a marker for BCR-ABL activity in CML patients.^{19,20}

Although the phosphorylation of CRK proteins is suppressed by ABL/BCR-ABL kinase inhibitors such as imatinib, drug resistance brought about by certain mutations such as the T315I gatekeeper mutation remains a major problem.²¹ Hence, inhibiting the binding of CRK with ABL kinase is an alternative way to overcome the imatinib resistance of BCR-ABL. We report here the structure-guided design of a high-affinity peptide inhibitor, PRM-3, for the interaction of

Scheme 1 Interaction between CRK-II and ABL kinase.

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CRK with ABL kinase. PRM-3 binds to the nSH3 domain of CRK-II and our *in vitro* assay shows that PRM-3 effectively inhibits the phosphorylation of CRK-II by wild-type ABL and T315I gatekeeper-mutant ABL kinases.

Results and discussion

We selected PRM-1 as a starting molecule for the optimization of binding affinity to the nSH3^{CRK} domain. The primary structure of PRM-1 was derived from C3G, which is one of the cellular binding partners of CRK-II.²² The K_d of nSH3:PRM-1 was reported to be 1.9 μ M (ref. 23) and its high-resolution crystal structure (PDB ID: 1CKA) was available for structure-based optimization.²³ Previous studies indicated that the nSH3 domain of CRK-II recognizes the core Px ϕ PxK sequence in PRMs, where ϕ and x represent hydrophobic and any amino acids, respectively.^{11,16,23} To describe the sequence of peptides used in this study, we adopted the notation proposed by Lim *et al.*,²⁴ where ϕ in the core sequence, Px ϕ PxK, is designated as the X₀ position (Table 1). The residues located at the C-terminal end of X₀ are designated as X₋₁, X₋₂, *etc.* The residues located at the N-terminal end of X₀ are designated as X₁, X₂, *etc.*

The crystal structure of the nSH3:PRM-1 complex showed that the N- and C-termini of PRM-1 are flexible owing to the lack of favorable interactions with the nSH3 domain.²³ To improve the interactions in the terminal regions of PRM-1, we designed PRM-2 with the following modifications: first, the sequence was extended by adding three residues to both N- and C-termini of PRM-1. The added residues were derived from the C3G sequence (residues 279–281) (Table 1). Second, P₋₂ in PRM-1 was replaced by K in PRM-2 to increase long-range electrostatic interactions as suggested by our previous study.²⁵ The structure of the nSH3:PRM-1 complex shows that the side chain of P₋₂ does not directly come into contact with the nSH3 domain, instead pointing away from the complex interface. Third, Y₋₇ was incorporated into the C-terminus for the spectroscopic quantitation of the peptide. Using the fluorescence change of W169 in the nSH3 domain upon binding to PRM-2, the K_d of the nSH3:PRM-2 complex was measured to be 0.4 μ M, which is 4-fold higher than that of PRM-1 (Fig. 1A).

To reveal the binding mode of PRM-2, we determined the high-resolution (1.77 Å) crystal structure of the nSH3:PRM-2 complex (PDB ID: 5L23) (Fig. 2A, Table S1, and Fig. S1†). The core sequence (PxLPxK) showed a virtually identical structure

between PRM-1 and PRM-2 (Fig. 2B and S2†). In contrast, the modified C-terminal residues (R₋₄-Q₋₅-S₋₆-Y₋₇) of PRM-2 showed a novel binding mode. The Ψ angle (-38.6°) of R₋₄ deviates significantly from that of the PPII helix ($\sim 150^\circ$) (Fig. 2C). Moreover, the aromatic side chain of the C-terminal Y₋₇ is sandwiched between the backbone of PRM-2 and the binding surface of the nSH3 domain (Fig. 2D). The side chain hydroxyl group of Y₋₇ is hydrogen-bonded (2.5 Å) to the side chain carboxyl group of E166 of the nSH3 domain, stabilizing the C-terminal residues of PRM-2. This binding mode has not been observed in the nSH3:PRM complexes.

The structure of the nSH3:PRM-2 complex showed a large negatively charged surface near the PRM-binding site in the nSH3 domain (Fig. 2E). This indicated that the addition of positively charged residues at the C-terminus of PRM-2 would enhance its long-range electrostatic interactions with negatively charged residues in the nSH3 domain. We therefore designed PRM-3 (Table 1) by adding two Arg residues to the C-terminus of PRM-2. In addition, three N-terminal residues of PRM-2 were removed because our crystal structure indicated that these residues lack favorable interactions with the nSH3 domain (Fig. 2A and S2†).

Remarkably, the binding affinity of PRM-3 to the nSH3 domain was too high to reliably measure it at 25 °C using intrinsic Trp fluorescence change. Therefore, we measured K_d values at three different higher temperatures and estimated the K_d at 25 °C using linear extrapolation of the temperature-dependent binding free energy (Fig. 1B and S3†). This yielded a K_d of ~ 9 nM at 25 °C, which is about 200-fold higher in affinity than that of PRM-1. Despite the large difference in K_d , NMR chemical shift perturbation analysis indicated that PRM-3 binds to the same site in the nSH3 domain as PRM-1 and PRM-2 (Fig. 3C).

To determine whether the increased binding affinity is due to enhanced long-range electrostatic interactions, we compared the K_d value of the nSH3:PRM-3 complex in the presence of 80 mM NaCl with that in the presence of 1 M NaCl (Fig. S3D†). The Debye screening lengths are 10.7 Å and 3.0 Å in the presence of 80 mM and 1 M NaCl, respectively. Hence, long-range electrostatic interactions are screened in a high-ionic strength solution, while the short-range electrostatic interactions within 3.0 Å remain effective under the same conditions. We observed that the K_d value of the nSH3:PRM-3 complex increased by 50-fold in the presence of 1 M NaCl compared to the one in the presence of 80 mM NaCl (Fig. S3D†). This result shows that the high binding affinity

Table 1 Binding affinities of PRMs to the nSH3 domain

PRMs	Sequences																	K_d (μ M) (uncertainty)
	7	6	5	4	3	2	1	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	
PRM-1				P	P	P	A	L	P	P	K	K	R					1.9 ^a
PRM-2	D	N	S	P	P	P	A	L	P	K	K	R	Q	S	Y			0.4 (+0.1, -0.1)
PRM-3				S	P	P	A	L	P	K	K	R	Q	S	Y	R	R	0.009 (+0.008, -0.004) ^b

^a Ref. 23. ^b The asymmetric uncertainty was estimated from the 95% confidence band of the linear regression curve.

Fig. 1 Fluorescence-based binding affinity measurements. (A) Binding isotherm of PRM-2 and the nSH3 domain. (B) Temperature-dependent binding free energy, ΔG_{bind} , of the nSH3:PRM-3 complex. The solid line represents the best linear fit to the corresponding data points. The dotted lines represent the 95% confidence band of the linear regression curve.

Fig. 2 Structural analysis of the nSH3:PRM-2 complex. (A) Crystal structure showing the binding mode of PRM-2. (B) Structural comparison of PRM-1 (PDB ID: 1CKA) and PRM-2 (PDB ID: 5L23) bound to the nSH3 domain. The ligands shown in magenta and green represent PRM-1 and PRM-2, respectively. For clarity, only the residues involved in key intermolecular interactions are shown as stick models. (C) Binding mode of the C-terminal R₄-Q₅-S₆-Y₇ moiety of PRM-2. The arrow represents the breakage of the PPII helix conformation at R₄. (D) The interaction between Y₇ of PRM-2 and E166 of the nSH3 domain. For clarity, the side chains of other residues are omitted. (E) Electrostatic potential surface of the nSH3 domain. Negatively and positively charged surfaces are shown in red and blue, respectively. PRM-2 is shown in green color.

of PRM-3 is largely due to the enhanced long-range electrostatic interactions with the nSH3 domain. The role of long-range electrostatic interactions in increasing binding affinity was also demonstrated in diverse protein-protein/ligand interactions.^{25–30}

To examine the uniqueness of the PRM-3 sequence, we searched for its sequence in known human proteome using ScanProsite.³¹ Interestingly, the search did not find a match for the sequence of PRM-3. The search without the last three modified amino acids (Y₇-R₈-R₉) retrieved C3G as the closest match. Although this does not directly indicate the selectivity of PRM-3 to the nSH3 domain of CRK-II, its unique sequence would be beneficial for reducing off-target effects in a cellular environment. Further cell-based selectivity tests will be conducted in our laboratory.

The interaction between the nSH3^{CRK} domain and PRM^{ABL} is a prerequisite step for the ABL-dependent phosphorylation

of CRK-II at Y221 (Scheme 1).^{11,17} We thereby tested the inhibitory activity of PRM-3 on ABL-dependent CRK-II phosphorylation using an *in vitro* assay with purified proteins. Upon phosphorylation, CRK-II undergoes a large-scale conformational change resulting in differential migration between CRK-II and CRKII-pY221 during native gel electrophoresis (PAGE) (Fig. 4A and B and S4†).^{32,33}

The assay showed that wild-type ABL kinase phosphorylated approximately 62% and 21% of CRK-II in the absence and presence of 1 μM PRM-3, respectively (Fig. 4A and C). Interestingly, the CRK phosphorylation level was ~33% in the presence of 1 μM imatinib under the same experimental conditions. This result shows that PRM-3 is a potent inhibitor for PPIs between CRK-II and ABL. Moreover, PRM-3 showed strong inhibition of CRK phosphorylation by the T315I gatekeeper mutant of ABL kinase (Fig. 4B and D), whereas imatinib was ineffective under the same conditions.

Fig. 3 Structural analysis of the nSH3:PRM-3 complex. (A) Overlaid ^1H - ^{15}N HSQC spectra of the free nSH3 domain (black) and the nSH3:PRM-3 (red) complex. The residues whose chemical shifts change significantly are labeled. (B) NMR chemical shift perturbation (CSP) of the nSH3 domain induced by binding of PRM-3. (C) Binding interface of the PRM-3 probed by the NMR CSP analysis. For comparison of the binding interface, PRM-2 is shown in green. The residues whose backbone chemical shifts are significant (larger than average +1 standard deviation) and modest (larger than average but less than average +1 standard deviation) are shown in magenta and orange, respectively.

Fig. 4 ABL-dependent CRK phosphorylation assay. Time-dependent phosphorylation of CRK-II by (A) wild-type and (B) T315I-ABL kinases in the absence (control) and presence of PRM-3 or imatinib. Plots of levels of CRK phosphorylation by (C) wild-type and (D) T315I-ABL kinases as a function of reaction time. Open circles represent the control. Blue and red circles represent the results in the presence of imatinib and PRM-3, respectively.

Mutations at the gatekeeper residues (T315I/M) have proven highly resistant to most drugs including imatinib and all second-generation tyrosine kinase inhibitors.

To overcome drug resistance, it is highly desirable to develop inhibitors, of which working mechanisms do not overlap with those of existing inhibitors. Our results highlight the

fundamental difference in the working mechanisms of imatinib and PRM-3: while imatinib binds to the active site of ABL/BCR-ABL, PRM-3 binds to CRK, which is a substrate of ABL/BCR-ABL. Therefore, it is likely that PRM-3 will also be effective for other kinase domain mutants of ABL or BCR-ABL, although further investigations using cell-based assays are needed.

In summary, we developed a highly potent peptide inhibitor, PRM-3, for the interaction between CRK-II and ABL kinase. PRM-3 binds to the nSH3 domain of CRK-II with a K_d of 9 nM, which is 200-fold higher in affinity than that of the starting molecule, PRM-1. We showed that the high affinity of PRM-3 is due to enhanced long-range electrostatic interactions. The mechanistic insights gained during the optimization of PRM-3 will facilitate the design of more potent and selective inhibitors for the CRK-mediated protein–protein interactions. The inhibitors will be useful for studying the role of CRK:ABL interactions in malignant behaviors of cancer cells.^{34–36}

Conflicts of interest

There are no conflicts to declare.

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