**ABL SH3 mutant inhibits BCR-ABL activity and increases imatinib sensitivity
by targeting RIN1 protein in CML cell**

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Abstract

SH3 domain plays an important role in maintaining autoinhibition of BCR-ABL protein. RIN1 interacts with BCR-ABL SH3 domain via PxxP motifs to promote autophosphorylation as well as activation of BCR-ABL tyrosine kinase, suggesting using exogenous SH3 domain which blocks the interaction of BCR-ABL and RIN1 could be an adjunct therapy for CML. Here, we reported a novel p-BCR-ABL inhibitor, designed as ABL SH3 mutant, and identified its effects on inhibiting the tyrosine kinase activity of BCR-ABL without or with imatinib (IM) *in vitro* and *in vivo*. Our results demonstrated that ABL SH3 mutant T79Y markedly repressed the expression of BCR-ABL signaling pathways in IM-resistant cell lines KCL22 and K562/G01 as well as IM-sensitive cell line K562. Moreover, combination of T79Y with IM considerably decreased the proliferation of leukemia cells *in vivo*. Inhibition of BCR-ABL and RIN1 interaction using exogenous modified BCR-ABL SH3 domain provides a feasible and alternative option of small molecule inhibitors for CML treatment.

Keywords: Chronic myeloid leukemia; BCR-ABL; SH3 domain; RIN1

1. Introduction

The oncoprotein BCR-ABL, which plays a vital role in the tumorigenesis of chronic myeloid leukemia (CML), is caused by the fusion of BCR with ABL N terminus. ABL is comprised of a N-terminal cap region, an Src-homology-2 (SH2), an SH3 domain, and a tyrosine kinase domain[1]. ABL SH3 domain binds to the SH2/kinase linker domain and forms a PPII helix to suppress c-ABL kinase activation[2-4]. Fusion of ABL to BCR disrupts the autoinhibitory ability of ABL and results in the constitutively activated tyrosine kinase activity [5]. Deletion, mutation, as well as alanine substitution of linker proline, activate the tyrosine kinase activity of ABL and cause resistance to imatinib (IM), which is one of the first cancer therapies to show the potential for targeted therapy[6, 7]. It has been reported that the binding specificity of an SH3 domain is determined by the amino acids in the flanking region of the core motif PxxP and Tyr-7, Asp-8, Asn-51 and Tyr-52 are reported to play a vital role in binding specificity[8], which suggests that blocking or strengthening of SH3 binding specificity by mutation of key amino acids can be used to regulate the kinase activity of BCR-ABL.

RIN1, as a RAS effector protein, contains a RAS association domain, a guanine nucleotide exchange factor (GEF) domain in the carboxy terminal section and tyrosine phosphorylation substrates, proline-rich domains in the amino terminal section[9]. RIN1 binds to PxxP motif in SH3 domain of ABL or BCR-ABL with low-affinity through its proline-rich region (PPAVPPPPVP)[10]. The interaction of RIN1 with ABL leads to phosphorylation of RIN1 on tyrosine 36, which subsequently associates with ABL SH2 domain. Activation of ABL by RIN1 is unaffected by an imatinib resistance mutation[11] and silencing of RIN1 suppresses the tyrosine phosphorylation of Crkl, one of ABL substrates. These observations demonstrate that RIN1 is both

a substrate and binding partner of ABL[12] and is required for maintaining normal ABL kinase activity. Blocking RIN1 and ABL interaction may have therapeutic implications in overcoming imatinib resistance.

To disable the interaction between RIN1 and BCR-ABL and thereby inhibit BCR-ABL kinase activation, we used computational modeling and simulation to design SH3 mutants which disrupting the interaction. We found one SH3 mutant, named T79Y, can directly bind to BCR-ABL and suppress phosphorylation of BCR-ABL. Combination of T79Y with IM is more effective at inhibiting the oncogenic activity of BCR-ABL *in vivo* than using IM alone.

2. Materials and methods

2.1 Computational modeling and simulation

The original model was acquired from the crystal structure of the ABL SH3 domain (Protein Data Bank code 3EG2, chain A, position 61-121 of whole ABL1), position 114 was mutated back to asparagine to keep the natural protein structure. Amino acid side chain mutations were introduced using PyMOL1.6, and molecular dynamics simulations were completed using NAMD 2.8 and VMD 1.8.7. The proline-rich motif (PPAVPPPPVP, residues 259-268, accession Q13671) of RIN1 was from UniProt database. Motif-protein binding sites on ABL-SH3 protein surfaces and specificity were predicted by PepSite2.0 online software (<http://pepsite2.russelllab.org/>)[13]. First, the key amino acids of motif-protein binding sites were tested by PepSite2.0, amino acids were visualized and detected by NAMD 2.8[14] and VMD 1.8.7[15], and the distribution of key amino acids on ABL-SH3 surface was found. Second, amino acids of this region were selectively mutated, and the mutants' structure were analyzed using NAMD 2.8 and VMD 1.8.7 referring to

the previous methods[16, 17]. Subsequently, the binding sites and the specificity of motif-mutants were tested by PepSite2.0.

2.2 Vectors, virus production and infection

Wild-type and mutated ABL SH3 domain were amplified by SOE-PCR as previously described[18] using pMig210 plasmid containing BCR-ABL p210 gene as a template. The Ad5/f35 adenoviral vector system was a gift from Dr. Tongchuan He. T79Y was cloned directly into HA-tagged pAdTrack-CMV shuttle vector using the SalI and XhoI sites. The construct was verified by DNA sequencing. Generation of Ad5/f35-GFP recombinant adenoviruses were performed as previously described [19]. KCL22, K562 and K562/G01 cells were infected with viruses and the transduction efficiency was evaluated by GFP expression. The expected molecular weight for T79Y was 7 kDa. The primers used were listed in *Table 1*.

2.3 Cell culture

Human BCR-ABL (p210)-positive CML cell lines KCL22, K562 and K562/G01 were cultured in RPMI-1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA). AD293 cells were cultured in DMEM (Gibco, USA) medium supplemented with 10% FBS. All cells were cultured in a humidified incubator at 37°C with 5% CO₂.

2.4 Immunofluorescence

Treated K562 cells were collected and fixed. After blocking in 10% normal goat serum for 1 h at 37°C, cells were incubated with mouse anti-HA tag primary antibody (Cell Signal, USA), followed by incubating with goat anti-mouse Cy3-IgG antibody (Molecular probes, Eugene, OR), staining with DAPI at room temperature, and examining using a Nikon fluorescent microscope Eclipse 80i (Melville, NY).

2.5 Western blot and immunoprecipitation

Treated cells were collected for protein extraction using protein extraction reagent supplemented with proteinase and phosphatase inhibitors (Cell Signal, USA). Proteins were resolved on 8-10% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, MA), and labeled with primary antibodies respectively, including anti- β -actin (Santa Cruz, USA), anti-p-Bcr (Cell Signal, USA), anti-c-Abl (Cell Signal, USA), anti-Akt and anti-p-Akt (Cell Signal, USA), anti-p-Erk (Cell Signal, USA), anti-Erk (Santa Cruz, USA), anti-Stat5 and anti-p-Stat5 (Cell Signal, USA), anti-Crk1 and anti-p-Crk1 (Bioworld, USA), anti-Bax (Cell Signal, USA), anti-Bcl-2 (Santa Cruz, USA), anti-Cyclin D1 (Santa Cruz, USA), anti-c-Myc (Cell Signal, USA), followed by HRP-conjugated anti-mouse, anti-goat or anti-rabbit antibodies (Santa Cruz, USA). Signals were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore Billerica, MA), imaged and analyzed by the Bio-Rad Gel Imaging System on cool image workstation II (Viagene, USA).

KCL22 cells were collected and lysed in NP-40 lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, and protease inhibitors), and following procedures were performed according to the immunoprecipitation kit instructions (Pierce, USA). Eluted samples were resolved by electrophoresis on 8% SDS-PAGE gel, followed by immunoblotting using anti-RIN1 antibody (Santa Cruz, USA).

2.6 Cell proliferation

Cells were seeded into 96-well plates at a density of 1,000 cells/ well and incubated for 24 h, 48 h and 72 h. Four replicate wells were used for each treated group. At the end of each time, 50 μ l MTT (2 mg/ml, Sigma) was added to each well and further incubated for 4 h at 37°C. MTT

solution was removed and the formazan crystals were dissolved in DMSO. Absorbance was read at 570 nm using a microplated reader.

2.7 Cell apoptosis

KCL22 and K562 cells were treated with T79Y combined with different concentrations of IM or PBS for 72 h. Cells were collected and fixed. Apoptosis was assessed by Annexin V-PE/7-AAD double-staining via an Apoptosis Detection Kit (Becton-Dickinson). Cells were resuspended with 500 µl binding buffer and stained with Annexin V-PE and 7-AAD for 15 min at room temperature in the dark. Signals were analyzed by flow cytometry. Moreover, Wright's staining was used to examine apoptotic cell morphology and results were observed under light microscope.

2.8 RNA isolation and semi-quantitative PCR

RNA was isolated using RNAiso Plus (Takara, China) according to manufacturers' instructions. 1 µg of RNA was converted to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, China). The produced cDNA was amplified with specific primers for human BCR-ABL (forward primer was 5'-GCAAGCTTACCATGGACATCCGTGG-3'; reverse primer was 5'-GTCGACCTTGCCATCAGAAGC-3') and β -actin (forward primer was 5'-CTGTCCCTGTATGCCTCTG-3'; reverse primer was 5'-ATGTCACGCACGATTTC-3') using Takara Ex Taq kit (Takara, China). Products were analyzed by agarose gels. The expression level of each gene was normalized using β -actin as a reference.

2.9 Mouse studies

KCL22 cells were cultured with or without T79Y in combination with IM for 72 h. Cells (2.0×10^7) were harvested, resuspended in PBS and injected intravenously into the tail vein of 5-6 week-old NOD/SCID mice. Mice were monitored weekly for signs of weight loss or lethargy.

Peripheral blood was obtained from mice and white blood cell counts were analyzed. Mice were killed after 13 weeks, and bone marrow cells were harvested from femurs for Wright's staining and PCR. The tissues of liver and spleen were analyzed by hematoxylin and eosin staining (HE staining). Kaplan-Meier plots were generated using Excel. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiments.

2.10 Statistical analysis

All experiments were repeated for three times and all values were expressed as means $\pm$ standard deviations. The results were analyzed with one-way ANOVA to compare the mean of each group with that of the control group. When *p-values* <0.05 , the difference between two means was considered statistically significant.

3. Results

3.1 Screening peptide with high affinity to RIN1

To design peptide with high affinity to RIN1 as compared with wild-type ABL SH3 domain, we used NAMD, which is a parallel molecular dynamics code designed for high-performance simulation of large biomolecular systems, as a platform to evaluate stability and gross motions in different operating conditions[14]. The Visual Molecular Dynamics (VMD) and PyMCL were used as the visualization software for analyzing trajectories generated by NAMD. The binding specificity between the newly designed peptide and RIN1 protein surface was predicted using PepSite2.0, and peptide sequences that bound to RIN1 with low *p-values* were obtained[20, 21].

Since RIN1 binds to SH3 domain of ABL through the proline-rich motif, we mutated or deleted several key aromatic amino acids surrounding the hydrophobic pocket of ABL SH3 domain. We selected 2 peptides (W99R and T79Y) with predicted outcomes better than wild-type ABL SH3 as candidate sequences (Fig. 1A and 1B).

3.2 Characterization and expression of the ABL SH3 mutants in an adenoviral expression system

We used adenoviral system to express the ABL SH3 mutants W99R and T79Y. As shown in Fig. 2A, we analyzed the interaction using HA pull-down and we didn't detect any binding of W99R to RIN1 *in vitro* which was consistent with our bioinformatics analysis. Therefore, we focused our study on T79Y which can directly bind to RIN1. Immunofluorescence assay suggested that T79Y was mostly localized in the cytoplasm of leukemia cells (Fig. 2B). Overexpression of T79Y suppressed phosphorylated BCR-ABL (Fig. 2C) and total BCR-ABL (Fig. 2D) protein expression in three CML cell lines K562, KCL22 and K562/G01. Crkl and Stat5 are two key substrates downstream of BCR-ABL and our data further confirmed that T79Y attenuated the levels of phosphor-Crkl and phosphor-Stat5 without affecting total protein level (Fig. 2D).

3.3 Cotreatment with T79Y and IM exerts synergistic apoptotic effect against leukemia cells

Next, we determined the effects of cotreatment with T79Y on IM-mediated proliferation and apoptosis of K562, IM-resistant K562/G01 and KCL22 cells. Cell viability was suppressed when treated with IM and T79Y (Fig. 3A, 3B and 3C), which suggested that T79Y promoted IM mediated cell growth inhibition. Moreover, combined treatment with T79Y significantly enhanced apoptosis induced by IM (Fig. 3D). This effect was also confirmed by Wright's staining that cells

treated with T79Y and IM demonstrated more significant nuclear condensation and apoptotic bodies (Fig. 3E).

3.4 Cotreatment with T79Y and IM exerts anti-BCR-ABL activity

We examined the effects of cotreatment with T79Y and IM on BCR-ABL activity in IM-sensitive and IM-resistant cell lines, as well as on the levels of the signaling proteins downstream of BCR-ABL. Our results showed that, combined of T79Y with IM caused more depletion of BCR-ABL, as compared with treatment with either agent alone. Combined treatment also caused more attenuation of levels of p-Crk1, p-AKT, p-ERK, MEK and p-Stat5 (Fig. 4). Following cotreatment with T79Y and IM, induction of BAX and attenuation of Bcl-2 were observed. The expression of c-myc and cyclin D1 was suppressed. Similar effects of T79Y and IM were observed in all three cell lines (Fig. 4).

3.5 T79Y potentiates the efficacy of IM in a CML mouse model

We compared the efficacy of combined use of IM and T79Y on the growth of tumors induced by KCL22 cells. Cells treated with PBS, IM or IM with T79Y were injected into mice through tail vein. Mice receiving PBS-treated KCL22 cells displayed the characteristics of high white blood cell counts, splenomegaly and hepatomegaly (Fig. 5A). In contrast, recipients of T79Y and IM treated KCL22 cells showed much lower WBC counts, spleen and liver weight (Fig. 5A-5D). The leukemic cell infiltrations in the bone marrow, liver and spleen were also examined. Mice with KCL22 cells pre-treated with T79Y and IM were found to have significantly reduced leukemic cell infiltration of bone marrow, liver and spleen (Fig. 5E). Survival of mice receiving T79Y and IM treated cells was significantly prolonged in comparison with control mice and IM treated mice (Fig. 5F). We also collected bone marrow cells from each group and examined the expression of

BCR-ABL gene. Our results showed that T79Y combined with IM completely, whereas IM alone only partially, abrogated the expression of BCR/ABL (Fig. 5G).

4. Discussion

The BCR-ABL oncogene results from the t(9;22)(q34;q11) reciprocal translocation and possesses high tyrosine kinase activity which activates downstream cellular signal pathways, such as JAK2/STAT5[22], PI3K/AKT[23] and RAS[24], leading to tumor cell proliferation. RIN1 protein directly binds to BCR-ABL and activates ABL kinase activity through reinforcing the activated conformation of ABL/BCR-ABL[25], and is considered to modulate the sensitivity of BCR-ABL positive CML cell lines to IM[11]. Since RIN1 binds to BCR-ABL with low affinity[26, 27], we proposed that an exogenous peptide with improved binding affinity to BCR-ABL can be used to disrupt the activation of BCR-ABL.

In the present study, we employed bioinformatics approaches to generate ABL SH3 mutants as inhibitors of the RIN1/BCR-ABL binding interaction. Bioinformatic approaches enable the prediction and selection of candidate sequences at a relatively low cost and high efficiency. Based on our *in silico* analysis, we selected two mutants (W99R and T79Y) for further study.

As shown in Fig. 2A, only T79Y can directly bind to RIN1. The other functional peptide W99R which showed higher p-value than wild-type SH3 as calculated by PepSite2.0 didn't bind to RIN1. It appears that other proteins may mediate the interaction of W99R with RIN1 (data not shown). More experiments need to be done to investigate how W99R abrogates BCR-ABL activity without binding to RIN1. Based on these results, we focused on the function of T79Y *in vitro* and *in vivo*.

The interaction of RIN1 to BCR-ABL SH2 and SH3 domain takes place in the cytoplasm. So, it is important to determine if the site of T79Y-RIN1 interaction is consistent with the localization of RIN1 and BCR-ABL. We therefore did immunofluorescence assay and found T79Y accumulated in the cytosol (Fig. 2B). Next, we examined the effects of IM in combination with T79Y in treating CML. Our results showed that T79Y dramatically enhanced the efficacy of IM, leading to reduction of leukemic cells infiltration and alleviate the load of leukemia cells in peripheral blood and bone marrow (Fig. 5A and 5E). In T79Y and IM pre-treated mice, the weight and size of spleen and liver were significantly less than IM treated alone or PBS treated mice (Fig. 5B-5D). Survival analysis also showed that combined treatment with IM and T79Y prolonged the lifespan of mice loaded with leukemia (Fig. 5F). PCR analysis of residue of BCR-ABL in the bone marrow suggests that KCL22 cells pre-treated with IM and T79Y cannot survive in mice (Fig. 5G), which was not observed in IM treated mice further confirmed that T79Y may be used in patients bearing IM resistance. An investigation of the molecular mechanisms underlying the *in vivo* and *in vitro* effects of IM and T79Y demonstrated that they act synergistically on BCR-ABL activity. We found that T79Y alone inhibited the expression of phosphor-BCR-ABL and BCR-ABL (Fig. 2). Combination of T79Y and IM further suppressed the cell growth rate and enhanced apoptosis as shown by flow cytometry analysis and Wright's staining (Fig. 3). T79Y treatment results in a significantly decrease of numerous signaling pathways in the presence of IM, including JAK/STAT, PI3K/AKT and Raf/MEK/ERK (Fig. 4). BCR-ABL is a constitutively activated tyrosine kinase that has many substrates and activates multiple signal pathways. The downstream targets of STAT5 responsible for enhanced survival of BCR-ABL positive cells are involved in the transcription of Bcl-2 and cyclin D1 [28, 29]. Treatment with T79Y and IM resulted in

downregulation of Bcl-2 and cyclin D1 and upregulation of Bax (Fig. 4). It has been shown that c-myc is required for BCR-ABL oncogenic transformation [30], T79Y combined with IM significantly repressed c-myc expression (Fig. 4).

It has been reported that RIN1 increases ABL catalytic site accessibility for a wide range of substrates and even ABL with T315I mutation remains responsiveness to RIN1[11], suggests that disruption of the interaction between RIN1 and ABL synergize with IM to reduce the rate of CML relapse. Here, our study proposed one novel peptide T79Y which abrogates the interaction of RIN1 and ABL resulting in inactivation of BCR-ABL. Combination of T79Y potentiates the efficacy of IM. Therefore, combining IM with peptides disrupting the ABL and RIN1 interaction can be explored as a promising anti-leukemia strategy for CML treatment.

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Figure legends

Figure 1 Analysis of ABL SH3 mutants using Bioinformatic approaches (A) *p* values (Y-axis) of mutated peptides designed according to residues 258 to 268 of the RIN1 protein. (B) Amino acids information for WT and mutant ABL SH3 domain. Mutated amino acids were highlighted in grey.

Figure 2 Characterization of ABL SH3 mutants (A) The interaction of RIN1 and ABL SH3 mutants T79Y and W99R was confirmed by anti-HA pull down. (B) KCL22 cell was treated with T79Y, PBS or virus control pAd-null for 72 hours and cells were collected for immunofluorescence analysis using HA antibody which detected the tag on pAdtrack-CMV vector. Cy3 (red) staining indicates T79Y localization. Scale bar, 20 μ m. (C) K562, KCL22 and K562/G01 cells were treated with T79Y for 72 h, and the activity of p-BCR-ABL was determined by western blot. (D) K562, KCL22 and K562/G01 cells treated with T79Y were collected for the expression of p-Crk1 and Crk1, p-Stat5 and Stat5. β -actin was used as a reference.

Figure 3 Cotreatment with T79Y and IM promote apoptosis of leukemia cell. K562 (A), K562/G01 (B) and KCL22 (C) cells were treated with T79Y with or without 3 μ M IM for 24 h, 48 h, 72 h, and cell growth ratio was evaluated by MTT assay. (D) The percentage of early apoptosis cells were examined by flow cytometry. (E) Treated cells were collected for Wright's staining. Cells treated with T79Y and IM showed significant characteristics of apoptosis (black arrow). Scale bar, 20 μ m. ** $p < 0.01$, * $p < 0.05$ versus IM alone.

Figure 4 Cotreatment with T79Y and IM suppress BCR-ABL signal pathway. K562, KCL22 and K562/G01 cells were treated with T79Y together with IM for 72 h, Bcl-2, Bax, c-Myc and Cyclin D1 activities, and the expression of p-BCR-ABL/BCR-ABL, p-Crk1/Crk1, p-Stat5/Stat5,

p-Akt/Akt and p-Erk/Erk was analyzed by western blot. Right panel showed quantification of the bands and β -actin was used as a loading control.

Figure 5 T79Y potentiates the efficacy of IM in a CML mouse model. KCL22 cells treated with IM, together with or without T79Y were transplanted into mice through tail vein. (A) Blood was collected from all the animals by tail vein bleeding 5 weeks after cell transplantation and total WBC count were determined. Mean liver (B) and spleen (C) weight of mice transplanted with KCL22 cells were quantified. (D) Representative images of spleens and livers from each group. (E) Representative images of histologic sections of liver and spleen and bone marrow cells from mice transplanted with KCL22 cells in the presence of indicated treatments. Sections were stained with hematoxylin & eosin. (F) Survival curves were shown by Kaplan-Meier methods. (G) Bone marrow cells were collected and the expression of BCR-ABL was determined by semi-quantitative PCR. * $p < 0.05$ versus control group, $\blacktriangle p < 0.05$ versus IM alone.

Figure 1

A

B

Residues	60	70	80	90	100	110
ABL SH3	MNEDPNLF	VALYDFVAS	GDNTLSITK	GEKLRVL	GYNHNGEW	CEAQTKNGQGWVPSNYITPVNS
W99R	MNEDPNLF	VALYDFVAS	GDNTLSITK	GEKLRVL	GYNHNGEW	CEAQTKNGQGWVPSNYITPVNS
T79Y	MNEDPNLF	VALYDFVAS	GDNTLSITK	GEKLRVL	GYNHNGEW	CEAQTKNGQGWVPSNYITPVNS

Figure 2

Figure 3

Figure 4

Figure 5

Table1 Primers for peptide inhibitors construction

Mutants	Primers Sequence
ABL1-SH3	F, 5'- GCG <i>TCGACA</i> AACTTTTCGTTGCACTGTATGATT-3'
	R, 5'-CCG <i>CTCGAGTC</i> ATGGCGTGATGTAGTTGCTTGG -3'
P1 (W99R)	Rm1, 5'-GGGCTTCACAC <i>Cg</i> TTCCCCATTGTG-3'
	Fm1, 5'-CAATGGGGAA <i>c</i> GGTGTGAAG-3'
P2 (T79Y)	Rm3, 5'-ATTCCCCATTGTGAATATAGCCTAAGACCC-3'
	Fm3, 5'-GTGGAGATAA <i>Ctat</i> CTAAGCATAACTAA-3'
The outside mutagenesis primers were consistent with the wild ABL1-SH3 gene	

The forward primer contains a *Sall* restriction site and the reverse primer contains an *XhoI* restriction site, the restriction site is indicated by bold italic letters, and the termination codon is emphasized in shade. The specific-mutation sites are indicated by bold lowercase letters.