Brazilian Journal of Medical and Biological Research (2014) 47(12): 1096-1101, http://dx.doi.org/10.1590/1414-431X20143734
ISSN 1414-431X

A combination of STI571 and BCR-ABL1 siRNA with overexpressed p15INK4B induced enhanced proliferation inhibition and apoptosis in chronic myeloid leukemia

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Abstract

p15INK4B, a cyclin-dependent kinase inhibitor, has been recognized as a tumor suppressor. Loss of or methylation of the *p15INK4B* gene in chronic myeloid leukemia (CML) cells enhances myeloid progenitor formation from common myeloid progenitors. Therefore, we examined the effects of overexpressed p15INK4B on proliferation and apoptosis of CML cells. Overexpression of p15INK4B inhibited the growth of K562 cells by downregulation of cyclin-dependent kinase 4 (CDK4) and cyclin D1 expression. Overexpression of p15INK4B also induced apoptosis of K562 cells by upregulating Bax expression and downregulating Bcl-2 expression. Overexpression of p15INK4B together with STI571 (imatinib) or BCR-ABL1 small interfering RNA (siRNA) also enhanced growth inhibition and apoptosis induction of K562 cells. The enhanced effect was also mediated by reduction of cyclin D1 and CDK4 and regulation of Bax and Bcl-2. In conclusion, our study may provide new insights into the role of p15INK4B in CML and a potential therapeutic target for overcoming tyrosine kinase inhibitor resistance in CML.

Key words: Apoptosis; Chronic myeloid leukemia; p15INK4B; STI571 (Gleevec, imatinib)

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease originating from a constitutively active tyrosine kinase, BCR-ABL1 (1). The BCR-ABL1 fusion protein is able to disorder the cell regulation system and confer malignant differentiation and proliferation of hematopoietic cells, thus directly contributing to leukemogenesis. Therefore, formation of the *BCR-ABL1* fusion gene is a key step in the pathogenesis of CML.

In recent years, inhibition of BCR-ABL1 with tyrosine kinase inhibitors (TKIs) or small interfering RNA (siRNA) has been demonstrated to be an efficient targeted therapy for CML in the chronic phase (2-5). The BCR-ABL1 tyrosine kinase inhibitor, STI571, now called imatinib, is widely used in the treatment of CML (6,7). STI571 may inhibit proliferation and induce apoptosis of CML cells (6). Exposure of K562 cells to morpholino oligo antisense targeted against BCR-ABL1 inhibited proliferation of K562 cells but did not induce apoptosis (3). Zaree Mahmodabady et al. (4) showed that silencing BCR-ABL1 by specific siRNA effectively induced apoptosis of K562 cells and reduced viability. Although TKIs and targeting of the *BCR-ABL1* fusion gene by siRNA have displayed unprecedented efficacy for the treatment of CML (8), there are also many shortcomings that limit the application of these therapeutic methods, such as transfection efficiency, toxicity, and drug resistance (9,10). As a single drug, STI571 has been shown to be ineffective because of drug resistance (11). Although second-generation TKIs such as AMN107 appear to be able to improve the treatment of CML, TKI resistance and relapse also occur frequently in patients (12).

In recent years, combined therapy has become a trend in the treatment of CML. Oh et al. (13) showed that a combination of simvastatin and imatinib exhibited a synergistic killing effect in imatinib-resistant CML cells (13). Ciarcia et al. (14) also reported that phosphatidylinositol 3-kinase (PI3K) and the proto-oncogene SRC kinase inhibitors interacted synergistically with imatinib by inducing apoptosis and autophagy in BCR-ABL1+ leukemia cells. P15INK4B is a cyclin-dependent kinase inhibitor encoded by the *CDKN2B* gene in humans. P15INK4B forms a complex with CDK4 or CDK6 to prevent the activation of CDKs, and thus functions as a cell growth regulator that inhibits cell

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Received January 26, 2014. Accepted April 30, 2014. First published online October 14, 2014.

cycle G1 progression. It has been shown that gene alteration of p15INK4B may play an important role in the progress of CML (15). *p15INK4B* is a downstream gene of the *BCR-ABL1* fusion gene (16). BCR-ABL1 may down-regulate p15INK4B mRNA and protein expression through the PI3K signaling pathway. BCR-ABL1 siRNA and STI571 can not only inhibit BCR-ABL1-induced p15INK4B down-regulation and also decrease protein degradation by suppressing the PI3K signaling pathway. Thus, the combination of p15INK4B and BCR-ABL1 inhibitors, TKI or specific siRNA, may be more effective in the treatment of CML. Interestingly, a previous study showed that imatinib, in combination with the *p15* gene, displayed an enhanced effect on the inhibition of K562 cell proliferation and promotion of its apoptosis (17). However, whether BCR-ABL1 siRNA could enhance the effect of p15INK4B or STI571 on proliferation and apoptosis of K562 cells, and the underlying mechanisms, has not been completely investigated. Therefore, we investigated the effects of p15INK4B, alone or in combination with BCR-ABL1 inhibitors, on proliferation and apoptosis of K562 cells, and we explored the mechanisms in this study.

Material and Methods

Construction of plasmid (pcDNA3.1-p15INK4B)

The CDCBP reference sequence was used for primer designing on Primer Premier 5.0 software, and restriction sites (*Hin*dIII and *Eco*RI) and protective bases were added to the 5′ end of the forward and reverse primers, respectively. The primer sequences were as follows: forward, 5′-GTA AGC TTA TGG CCA CGT CTC TGG ATT TTA-3′; and reverse, 5′-TGG AAT TCT TAA CTA CTA GAC CAA TCT TGA-3′. The estimated length of the product was 465 bp. Total RNA was isolated from human peripheral blood mononuclear cells with TRIzol reagent (Invitrogen, USA), and cDNA was synthesized with a first-strand synthesis kit (TaKaRa, China). PCR reaction was performed with the GeneAmp PCR System 9700 (ABI, USA) and started with a polymerase activation step at 94°C for 3 min followed by 30 cycles at 94°C for 45 s, 55°C for 40 s, and 72°C for 60 s, and a final extension at 72°C for 30 min. The amplified gene with restriction sites was then cloned in mammalian expression vector pcDNA3.1. The constructed pcDNA3.1-p15INK4B plasmid was confirmed through PCR, restriction digestion, and sequencing.

Cell culture and plasmid transfection

The human leukemia cell line K562 (CML) was obtained from American Type Culture Collection (USA). Cells were maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin. K562 cells were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. All experiments were performed using cells in logarithmic growth phase.

K562 cells stably expressing p15INK4B (K562-p15INK4B) were constructed by transfection of pcDNA3.1-p15INK4B plasmid with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 48 h of transfection, cells were treated with G418, initially with 500 μg/mL for selecting stable clones then after 14 days with 200 μg/mL. The medium was changed every 72 h. Colonies of G418-resistant cells were selected and used in the growth inhibition assay.

Growth inhibition assay

Growth inhibition of K562 cells was examined using cell-counting kit-8 (Dojindo Laboratories, Japan). Briefly, 1×10^4 cells were seeded on 96-well plates. After 24 h, the medium was replaced with medium containing STI571 (5 μmol/L). After 24, 48, 72, 96, and 120 h of incubation, 10 mL of cell-counting kit solution was added and incubated for 2 h in a CO_2 incubator. The absorbance of each well was measured with a microplate reader at 450 nm. Means and standard deviation (SD) were generated from three independent experiments. Absorbance values were normalized to the values obtained from the control group to determine the value for percentage survival. Each assay was performed in triplicate.

Apoptosis assay

Apoptosis of K562 cells was analyzed by flow cytometry using an Annexin V/PI apoptosis kit according to the manufacturer's instructions (Key Technology, Ltd., China). K562 cells were plated onto 6-well plates (5×10^5 cells/well). The cells, after being treated with STI571 (5 μmol/L) for 24 h, were washed with phosphate-buffered saline (PBS) 3 times. They were then resuspended in 500 μL binding buffer and 5 μL Annexin V, and then 2 μL PI was added. Finally, the cells were analyzed using flow cytometry.

Western blot analysis

Western blot analysis was carried out as described previously (18). Briefly, total protein was isolated with radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) from K562 cells, and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime). Equal amounts of protein were separated by 10% SDS-PAGE under denaturing and nonreducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST) at room temperature for 1 h and then incubated with primary antibody at 4°C overnight. After they were washed in TBST, the blots were incubated with horse-radish-coupled secondary antibody. The signal was detected using enhanced chemiluminescence and recorded on X-ray film. The relative density of target bands was quantified using the Labworks 4.6 image acquisition and analysis software.

Statistical analysis

Data are reported as means ± SD. Statistical analysis was performed using ANOVA followed by Fisher's protected least significant differences test, and P<0.05 was considered to be statistically significant.

Results

Overexpression of p15INK4B alone or in combination with BCR-ABL1 inhibitors inhibited proliferation of K562 cells

As shown in Figure 1A and B, overexpression of p15INK4B, BCR-ABL1 siRNA, and STI571 significantly inhibited the proliferation of K562 cells after treatment for indicated times (P<0.01). The cell numbers in p15INK4B combined with BCR-ABL1 siRNA or STI571 groups were less than those in p15INK4B, BCR-ABL1 siRNA, and STI571 groups alone (P<0.01). Similarly, STI571 combined with BCR-ABL1 siRNA also showed an enhanced proliferation inhibition effect on K562 cells than when used alone (P<0.01; Figure 1C).

Overexpression of p15INK4B alone or in combination with BCR-ABL1 inhibitors induced K562 cells apoptosis

Next, we further investigated the effect of overexpression of p15INK4B alone or in combination with BCR-ABL1 inhibitors on the apoptosis of K562 cells by flow cytometry. BCR-ABL1 siRNA and STI571 induced an increase in apoptosis of K562 cells compared with control (P<0.01; Figure 2). Although p15INK4B overexpression increased the apoptosis rate of K562 cells compared with vector, there was no significant difference between the control and vector groups. When the cells with overexpressed p15INK4B were treated with BCR-ABL1 siRNA or STI571, apoptosis rates were further increased compared with BCR-ABL1 siRNA or STI571 alone (P<0.01). When BCR-ABL1 siRNA was used combined with STI571, the apoptosis rate was also higher than when used alone (P<0.01).

Effect of overexpression of P15INK4B alone or in combination with BCR-ABL1 inhibitors on cell cycle and apoptosis-related pathways

To explore the mechanisms underlying the effects of p15INK4B, BCR-ABL1 siRNA, and STI571 on proliferation and apoptosis of K562 cells, we analyzed apoptosis- and proliferation-related protein expression using Western blot analysis. As shown in Figure 3, p15INK4B, BCR-ABL1 siRNA, and STI571 significantly inhibited expression of cyclin D1 and CDK4. P15INK4B, in combination with BCR-ABL1 siRNA or STI571, further decreased levels of cyclin D1 and CDK4. P15INK4B, BCR-ABL1 siRNA, and STI571 also significantly upregulated expression of the proapoptotic protein Bax, whereas it downregulated expression of the anti-apoptotic protein Bcl-2. P15INK4B, in combination with BCR-ABL1 siRNA or STI571, also enhanced their effects on expression of Bax and Bcl-2.

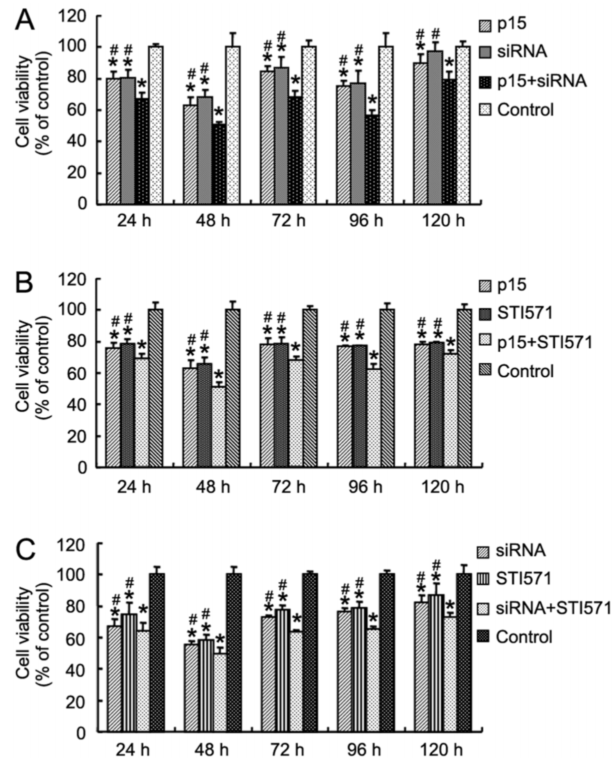

Figure 1. Effect of overexpressed p15INK4B alone or in combination with STI571 and BCR-ABL1 siRNA on the proliferation of K562 cells. *A*, Effect of p15INK4B in combination with BCR-ABL1 siRNA on the proliferation of K562 cells. BCR-ABL1 siRNA was transiently transfected into K562-p15INK4B cells and K562 cells transfected with pcDNA3.1 (K562/vector) with Lipofectamine 2000. After 24-h incubation, the cells were seeded on 96-well plates with complete RPMI 1640 medium at a density of 1×10^4 cells/well. K562/vector cells transfected with negative siRNA were used as control. MTT assay was performed after incubation for indicated times. *B*, Effect of p15INK4B in combination with STI571 on the proliferation of K562 cells. K562-p15INK4B cells were seeded on 96-well plates with complete RPMI 1640 medium at a density of 1×10^4 cells/well. K562/vector cells were used as control. After treatment with or without STI571 (5 μmol/L) for indicated times, MTT assay was performed. *C*, Effect of STI571 in combination with BCR-ABL1 siRNA on the proliferation of K562 cells. BCR-ABL1 siRNA was transiently transfected into K562 cells with Lipofectamine 2000. After 24 h, the cells were seeded on 96-well plates with complete RPMI 1640 medium at a density of 1×10^4 cells/well. K562 cells transiently transfected with negative siRNA were used as control. After treatment with or without STI571 (5 μmol/L) for indicated times, MTT assay was performed. Data are reported as means ± SD (n = 6). *P<0.01, compared with control; #P<0.01, compared with combination group (ANOVA followed by Fisher's protected least significant difference test).

Discussion

Drug resistance is a major obstacle to the successful treatment of CML patients with STI571. Novel agents or drug combinations that overcome this problem are eagerly

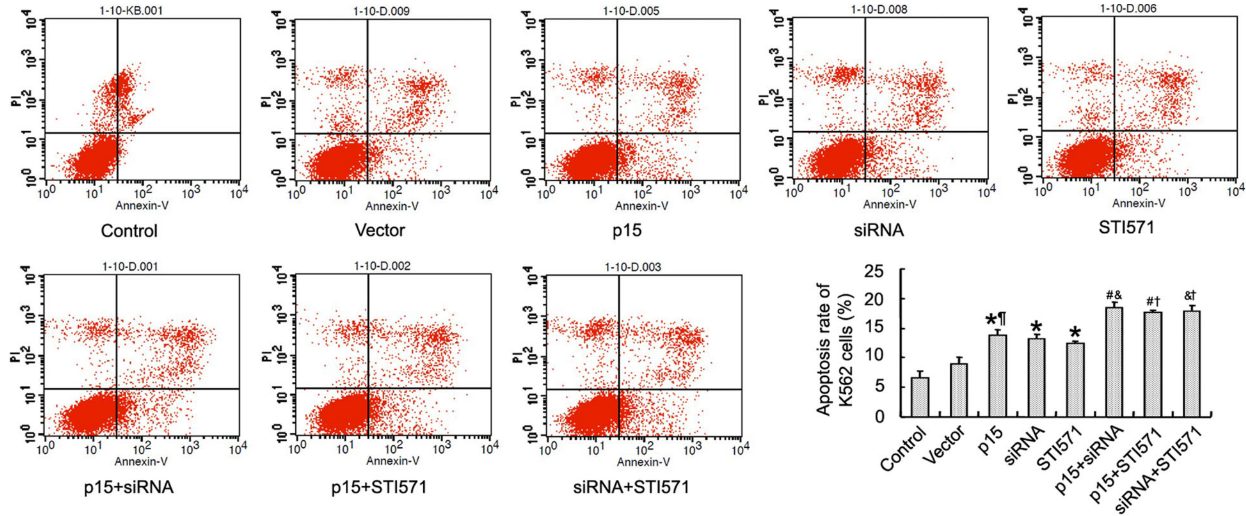

Figure 2. Effect of overexpressed p15INK4B alone or in combination with STI571 and BCR-ABL1 siRNA on the apoptosis of K562 cells. K562 cells were transiently transfected with p15INK4B, BCR-ABL1 siRNA or p15INK4B + BCR-ABL1 siRNA. After 24 h, the cells were plated onto 6-well plates at a density of 1×10^6 cells/well and incubated for another 24 h. Then, the cells were treated with STI571 (5 μmol/L) for 24 h and flow cytometry analysis was performed to determine the apoptosis of cells. Data are reported as means ± SD from 3 independent experiments. *$P<0.01$, compared with control; ¶$P<0.01$, compared with vector group; #$P<0.01$, compared with p15 group; &$P<0.01$, compared with BCR-ABL1 siRNA group; †$P<0.01$, compared with STI571 group (ANOVA followed by Fisher's protected least significant difference test).

sought. In the present study, our data showed that overexpressed p15INK4B in K562 cells significantly enhanced the effects of proliferation inhibition and apoptosis induction of BCR-ABL1 siRNA and STI571. In addition, *in vitro* treatment of K562 cells with combined BCR-ABL1 siRNA and STI571 was more effective in inhibiting proliferation and inducing apoptosis than any one used alone. Western blot analysis showed that overexpressed p15INK4B alone or in combination with STI571 and BCR-ABL1 siRNA increased Bax expression and decreased Bcl-2 expression in K562 cells. Overexpressed p15INK4B alone or in combination with STI571 and BCR-ABL1 siRNA also inhibited the expression of cell cycle related protein cyclin D1 and CDK4.

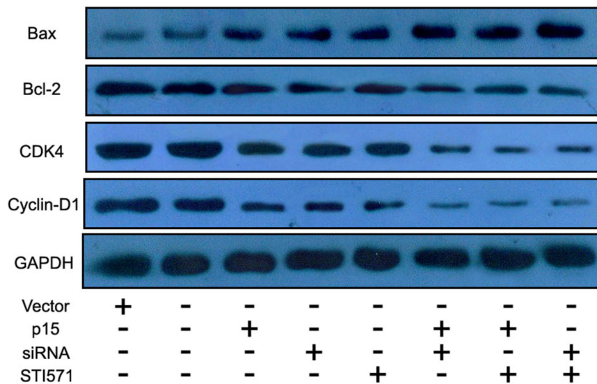

Figure 3. Effect of overexpressed p15INK4B alone or in combination with STI571 and BCR-ABL1 siRNA on cell cycle- and apoptosis-related proteins in K562 cells. K562 cells were transiently transfected with p15INK4B, BCR-ABL1 siRNA or p15INK4B + BCR-ABL1 siRNA. After 24 h, the cells were plated onto 6-well plates at a density of 1×10^6 cells/well and incubated for another 24 h. Then, the cells were treated with STI571 (5 μmol/L) for 24 h and Western blot was performed.

Imatinib mesylate has been widely used in the treatment of CML. However, it is becoming clear that a significant portion of patients with long-term imatinib treatment develop drug resistance because of the acquisition of mutations in the kinase domain of BCR-ABL1, which leads to an escape from treatment response (19). p15INK4B is an important inhibitor of CDK4 and CDK6, which play a critical role in the regulation of G(1)-S-phase transition of the cell cycle (20). It has been reported that p15INK4B functions independently as a tumor suppressor for myeloid leukemia development (21). Loss of p15INK4B enhances myeloid progenitor formation from common myeloid progenitors (22). Since the methylation of the p15INK4B promoter is a major gene silencing mechanism in CML (16), restoring the normal expression of p15INK4B should be an effective method in the treatment of CML. Consistent with a previous study (17), our results demonstrated that K562 cells with p15INK4B overexpression showed a lower proliferation rate and higher apoptosis rate than K562/vector cells, indicating that p15INK4B may be a therapeutic target for CML. We also found that overexpression of p15INK4B in combination with BCR-ABL1 siRNA or STI571 obviously increased the rate of proliferation inhibition and rate of apoptosis of K562 cells

compared with when used alone. These results indicate that recovery of p15INK4B expression may be a feasible method to overcome TKI resistance in the treatment of CML. Moreover, our study also demonstrated that the combination of BCR-ABL1 siRNA and STI571 displayed a stronger capacity for proliferation inhibition and apoptosis induction of K562 cells than BCR-ABL1 siRNA and STI571 used alone, which suggests that inhibiting both the transcription of the *BCR-AB1* fusion gene and the activity of its coded protein may be more effective than BCR-ABL1 siRNA or STI571 used alone.

Cyclin D1/CDK4 promotes G(1)-S-phase transition. Negatively regulating the p15INK4B-cyclin D1/CDK4-RB1-mediated pathway is one of the most common and important mechanisms in the growth advantage of tumor cells (23). In the present study, we showed that expression of cyclin D1 and CDK4 in K562 cells with overexpressed p15INK4B was obviously decreased compared with K562 cells transfected with vector, indicating that transfection of p15INK4B reactivates the p15INK4B-cyclin D1/CDK4-RB1 pathway and thereby inhibits proliferation of K562 cells. Besides, STI571 can also inhibit expression of cyclins, such as cyclin D1 and cyclin E. Therefore, we further observed the effect of overexpressed p15INK4B in combination with BCR-ABL1 siRNA or STI571 on cyclin D1 and CDK4 expression in K562 cells. The results showed that the combination of p15INK4B, BCR-ABL1 siRNA, and STI571 exhibited enhanced inhibition of cyclin D1 and CDK4 expression, which may account for the enhanced effect on proliferation of K562 cells. The anti-apoptotic protein Bcl-2 and proapoptotic protein Bax are important apoptosis-associated regulators in the mitochondrial apoptotic pathway (24). Levels of Bcl-2 and Bax may influence the sensitivity of cells to apoptotic stimuli. Thus, the alteration of their expression levels is of great importance in regulating apoptosis (25-27). Our study showed that p15INK4B overexpression, BCR-ABL1 siRNA, and STI571 all increased Bax expression and decreased Bcl-2 expression when used alone. The combination of overexpressed p15INK4B with BCR-ABL1 siRNA and STI571 also enhanced the regulation of Bax and Bcl-2 expression, resulting in an increase in apoptosis of K562 cells.

In conclusion, our data demonstrated that recovery of p15INK4B expression in CML cells inhibited cell proliferation and induced apoptosis. The combination of p15INK4B overexpression with BCR-ABL1 siRNA and STI571 could enhance cell proliferation and apoptosis due to the regulation of cell cycle- and apoptosis-related proteins. Our study may provide new insight into the role of p15INK4B in CML and a potential therapeutic method to overcome TKI resistance in CML.

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