

Brief report

Specific inhibition of *bcr-abl* gene expression by small interfering RNA

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Small interfering RNAs (siRNAs) were designed to target the *bcr-abl* oncogene, which causes chronic myeloid leukemia (CML) and *bcr-abl*-positive acute lymphoblastic leukemia (ALL). Chemically synthesized anti-*bcr-abl* siRNAs were selected using reporter gene constructs and were found to reduce *bcr-abl* mRNA up to 87% in *bcr-abl*-positive cell lines and in

primary cells from CML patients. This mRNA reduction was specific for *bcr-abl* because *c-abl* and *c-bcr* mRNA levels remained unaffected. Furthermore, protein expression of BCR-ABL and of laminA/C was reduced by specific siRNAs up to 80% in *bcr-abl*-positive and normal CD34⁺ cells, respectively. Finally, anti-*bcr-abl* siRNA inhibited BCR-ABL-depend-

ent, but not cytokine-dependent, proliferation in a *bcr-abl*-positive cell line. These data demonstrate that siRNA can specifically and efficiently interfere with the expression of an oncogenic fusion gene in hematopoietic cells. (Blood. 2003; 101:1566-1569)

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Introduction

RNA interference (RNAi) describes a highly conserved regulatory mechanism that mediates sequence-specific posttranscriptional gene silencing initiated by double-stranded RNA (dsRNA).¹⁻³ The RNase III enzyme Dicer processes dsRNA into approximately 22-nucleotide (nt) small interfering RNAs (siRNAs)⁴ that serve as guide sequences to induce target-specific mRNA cleavage by the RNA-induced silencing complex RISC.⁵ In plants and *Caenorhabditis elegans*, RNAi may involve the amplification of dsRNA by an RNA-dependent RNA polymerase (RdRP),⁶ and it enables systemic, long-term, and heritable gene silencing. In contrast, RNAi in *Drosophila* and mammals seems cell autonomous, transient, and nonheritable. Because exogenous application of siRNAs can efficiently trigger RNAi in mammalian cells,^{7,8} siRNAs are increasingly used in transient (co)transfection assays to modulate gene expression in mammalian cells, including human cells.⁹⁻¹²

Fusion transcripts encoding oncogenic proteins may represent potential targets for a tumor-specific RNAi approach. The Philadelphia (Ph) translocation t(9;22)(q34;q11) generates the *bcr-abl* fusion gene characteristic for chronic myelogenous leukemia (CML) and Ph⁺ acute lymphoblastic leukemia (ALL).¹³ Bcr-abl encodes a constitutively active cytoplasmic tyrosine kinase that is necessary and sufficient to induce and maintain leukemic transformation.¹⁴⁻¹⁶

We demonstrate that anti-*bcr-abl* siRNAs specifically inhibit *bcr-abl* mRNA expression in hematopoietic cell lines and primary CML cells. They reduce BCR-ABL protein expression and inhibit BCR-ABL-dependent, but not cytokine-dependent, cell proliferation. Therefore, anti-*bcr-abl* siRNAs may allow further analysis of BCR-ABL functions and, eventually, may lead to RNAi-based therapeutics.

Study design

siRNAs

Twenty-one-nucleotide single-stranded RNAs directed against the fusion sequence of *bcr-abl* were chemically synthesized (BioSpring, Frankfurt, Germany) (Figure 1A). The sense and antisense sequences were: b3a2_1, 5'-GCAGAGUUCAAAAGCCCUdTdT-3'; b3a2_3, 5'-AGCAGAGUUCAAAAGCCCUdTdT-3'; and b3a2_1, 5'-AAGGGCUUUUGAACUCUGCdTdT-3'; b3a2_3, 5'-AGGGCUUUUGAACUCUGCdTdT-3', respectively. Control anti-GL2_ and invGL2_siRNAs targeting GL2-luciferase, anti-laminA/C siRNAs, and siRNA duplexes were generated as described by Elbashir et al.⁷

Transfection of hematopoietic cells

K562, murine TonB cells derived from the BaF3 cell line, and primary hematopoietic cells were cultured as described.¹⁷⁻¹⁹ Cells (1 × 10⁶) were electroporated (330 V, 10 ms) in 100 μL RPMI 1640/10% fetal calf serum (FCS) containing 0.5 μg siRNA duplex in a 4-mm electroporation cuvette using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany).

Real-time RT-PCR

Real-time Taqman reverse transcription-polymerase chain reaction (RT-PCR) was performed as described^{20,21} with the probes and primers: bcrFP, 5'-AGCACGGACAGACTCATGGG-3'; bcrRP, 5'-GCTGCCAGTCTCTGTCCTGC-3'; bcr-Taqman-probe, 5'-AGGGCCAGGTCCAGCTGGA-CCC-3' covering the exon b5/b6 boundary; ablFP, 5'-GGCTGTCCCTCGTCTCCAG-3'; ablRP, 5'-TCAGACCCTGAGGCTCAAAGT-3'; abl-Taqman-probe, 5'-ATCTGGAAGAAGCCCTTCAGCGGC-3' covering the exon 1a/2 boundary. RNA from murine TonB cells was digested with DNase I and analyzed using the probe and primers: GAPDHmu 5', 5'-CAACAGGGTGGTGGACCTC-3'; GAPDHmu3', 5'-GGGTGGTCCAGGGTTTCTTA-3'; and GAPDHmu-Taqman probe, 5'-TGGCCTACATGGCCTCAAAGGA-3'.

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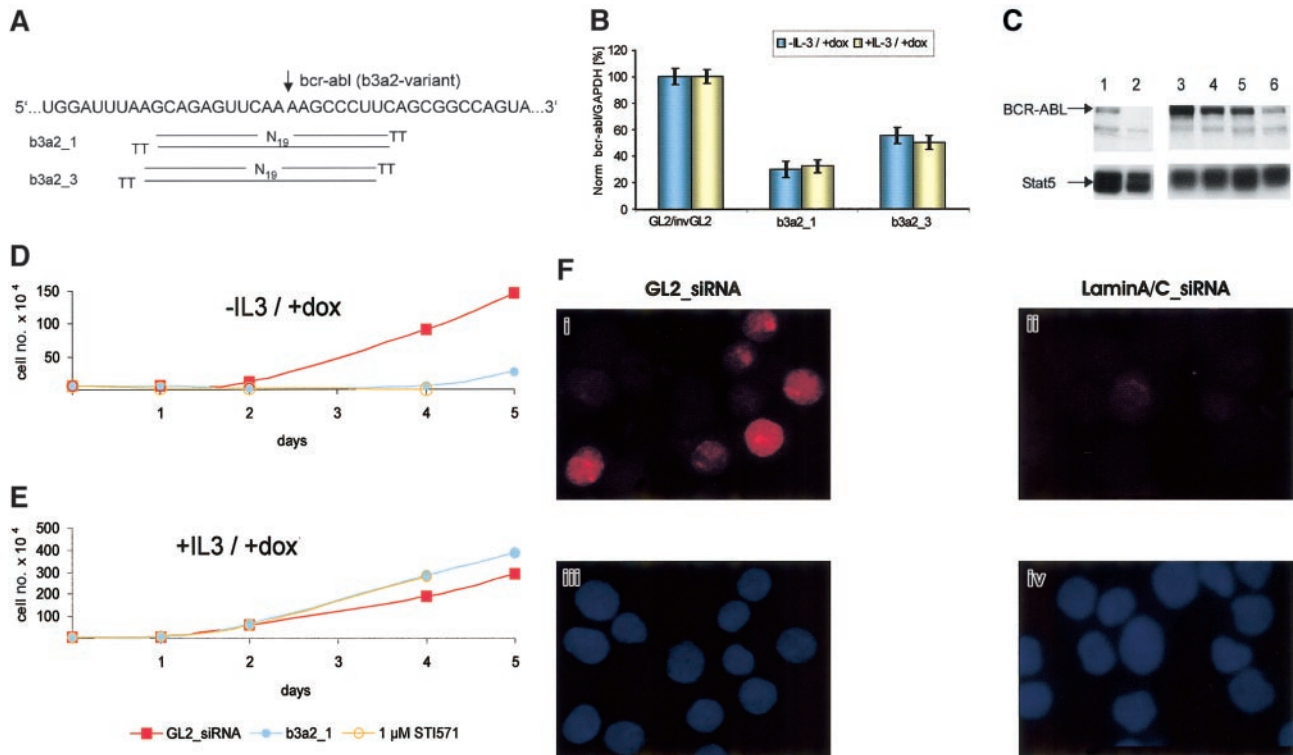


Figure 1. RNA interference in hematopoietic cells. (A) *Bcr-abl* fusion sequence and schematic representation of b3a2_1 and b3a2_3 siRNAs shifted by only one nucleotide. TT indicates the deoxythymidine dimer as 3' overhang. The arrow marks the fusion between *bcr* (left) and *abl* (right) sequences of the b3a2-*bcr-abl* variant. (B) siRNA mediated the reduction of *bcr-abl* mRNA expression in TonB cells in the presence or absence of IL-3. Normalized *bcr-abl*/GAPDH mRNA levels were measured 24 hours after electroporation and are shown compared with control cells treated with GL2/invGL2 control siRNAs (100%). The data represent mean \pm SD from 3 independent experiments. (C) Immunoblot of TonB cells treated with doxycycline and siRNAs. TonB cells were induced to express BCR-ABL (lanes 1, 3-6) or not (lane 2) by the addition of doxycycline at 1 μ g/mL. Cells were electroporated with b3a2_1 siRNA (lanes 4, 6) or control GL2_siRNA (lanes 3, 5) and were lysed 24 (lanes 3-4) and 60 hours (lanes 5-6) after electroporation, respectively. The upper panel shows an immunoblot with anti-BCR-specific antibodies, and the lower panel shows the same membrane reprobed with anti-Stat5 antibodies as loading control. (D-E) Effects of siRNAs on BCR-ABL-mediated (D) and IL-3-mediated (E) cell proliferation. TonB cells were either electroporated with control GL2_ (red squares) or b3a2_1 (blue-filled circles) siRNA or were left untreated in cultures containing 1 μ M STI571 (orange open circles). Viable cells were counted by trypan blue exclusion during suspension cultures after the addition of doxycycline at 1 μ g/mL without (D) or with (E) murine IL-3. Cell numbers of b3a2_1- and STI571-treated cells were nearly identical in the presence or absence of IL-3. (F) Inhibition of laminA/C protein expression by siRNAs in normal CD34⁺ cells. Normal CD34⁺ cells were electroporated with control GL2_ (i,ii) or anti-laminA/C siRNAs (ii,iv). Panels i-ii show immunostaining of laminA/C, and panels iii-iv show nuclear chromatin staining with DAPI (4',6'-diamidino-2-phenylindole-2HCl) stain.

Immunoblotting and immunofluorescence microscopy

Cellular lysates from TonB cells were immunoblotted with polyclonal anti-*bcr* antibody (N-20) and polyclonal anti-Stat5 antibody (C-17) as described.¹⁸ To quantify transduction efficacy, laminA/C immunostaining was performed using a monoclonal anti-laminA/C antibody (sc-7292; all antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA) as described.⁷

Primary normal and CML cells

Normal and CML CD34⁺ cells were purified to 95% or more, as described.¹⁹ Primary CD34⁺ cells or peripheral blood-derived mononuclear cells (PBMNCs) were cultured in X-VIVO/1% human serum albumin (HSA) with recombinant human stem cell factor (SCF; 100 ng/mL), Flt-3-ligand (100 ng/mL), and thrombopoietin (TPO; 20 ng/mL) before electroporation, and granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) (10 ng/mL each) were added thereafter. Methylcellulose colony assays were performed as described.¹⁹

Results and discussion

Selection of anti-*bcr-abl* siRNAs

Efficient siRNAs targeted against the b3a2-fusion sequence of *bcr-abl* were selected by cotransfection with a chimeric *bcr-abl*-

enhanced green fluorescence protein (EGFP) reporter gene in HeLa cells. HeLa cells could be transfected up to 95% (data not shown). Of 4 chemically synthesized 21-nt siRNAs tested, b3a2_1 and b3a2_3 (Figure 1A) were the most efficient. They reduced the number of fluorescent cells (to 90%), *bcr-abl*-EGFP mRNA levels (to 87%), and fluorescence intensity per cell (up to 100-fold) 24 hours after transfection (data not shown). RNAi was specific because no reduction in fluorescence intensity was found with control siRNAs or when native EGFP without *bcr-abl* sequences was used as reporter. Furthermore, anti-b3a2-*bcr-abl* siRNA only reduced the b3a2-, but not the b2a2-, variant of a *bcr-abl*-EGFP reporter gene and vice versa (data not shown).

Effects of siRNAs in hematopoietic cell lines

The siRNAs b3a2_1 and b3a2_3 were tested in Ph⁺ K562 and TonB cells expressing *bcr-abl* under the control of a doxycycline-inducible promoter.¹⁷ Transfection efficacy was analyzed using the laminA/C system and reached approximately 80% in K562 cells (data not shown). After 24 and 48 hours, b3a2_1 and b3a2_3, but not control siRNAs, reduced *bcr-abl* mRNA levels up to 24.8% and 35.2% (b3a2_1) and 32.4% and 61% (b3a2_3), respectively (Tables 1 and 2 and data not shown). Notably, c-*bcr* and c-*abl* mRNA levels remained unaffected, demonstrating the

specificity of anti-bcr-abl siRNAs (Tables 1-2). Furthermore, b3a2_1 and b3a2_3 transiently reduced the number of viable K562 cells by 75% in suspension cultures 4 days after electroporation (data not shown).

Cultures of TonB cells with or without doxycycline and IL-3 allow separate studies of BCR-ABL- and IL-3-mediated cell proliferation, with factor-independent proliferation considered as a surrogate marker for cellular transformation. After electroporation with b3a2_1 siRNA, bcr-abl mRNA declined by approximately 70% independent of IL-3 (Figure 1B). Furthermore, b3a2_1 siRNA reduced BCR-ABL protein expression by approximately 55%, as analyzed by immunoblotting and densitometry (Figure 1C). Finally, b3a2_1 siRNA reduced the number of viable TonB cells to an extent similar to that for the selective tyrosine kinase inhibitor STI571 in the absence, but not in the presence, of IL-3 (Figure 1D-E). Again, cultures of TonB cells treated with b3a2_1 resumed growth after 3 to 5 days in all conditions tested. In parental bcr-abl-negative BaF3 cells, siRNA had no effect on cell proliferation (data not shown).

siRNA activity in primary hematopoietic cells

siRNA efficacy in primary hematopoietic cells was first demonstrated in normal CD34⁺ cells using the laminaA/C system. Anti-laminaA/C siRNA reduced laminaA/C protein expression by approximately 70% (Figure 1F). Next, electroporation of PBMNCs or purified CD34⁺ cells from 6 CML patients with b3a2_1 siRNA reduced bcr-abl mRNA levels by 50% to 79% compared with control siRNA (100%) (Table 1). Again, c-bcr and c-abl mRNA levels remained unchanged in both CML samples studied. When primary CML cells were transfected with b3a2_1 siRNA and grown in cytokine-supplemented liquid or semisolid cultures, no significant inhibition of cell proliferation or colony formation was observed (Table 2 and data not shown). In contrast, STI571 markedly reduced the number of viable cells in suspension cultures and the colony number derived from purified CD34⁺ cells, as shown in earlier studies²² (Table 2 and data not shown).

Table 1. Effects of anti-bcr-abl siRNAs on bcr-abl mRNA expression in bcr-abl-positive cells

Cell sample/siRNA	bcr-abl/GAPDH, %	c-bcr/GAPDH, %	c-abl/GAPDH, %
K562 cells			
GL2	99.7 ± 2.1	82.0 ± 13	97.5 ± 2.5
GL2_inv	98.0 ± 2.1	116 ± 13	102 ± 2.5
b3a2_1	24.8 ± 3.7	98.0 ± 2.5	95.0 ± 10
b3a2_3	32.4 ± 1.2	99.5 ± 2.2	109 ± 1.5
CML-PBMNCs			
GL2	100	ND	ND
b3a2_1			
No. 1	21	ND	ND
No. 2	45	ND	ND
No. 3	46	ND	ND
No. 4	45	ND	ND
CML-CD34⁺ cells			
GL2	100	100	100
b3a2_1			
No. 5	22	102	98
No. 6	50	104	92

The data represent normalized bcr-abl, c-bcr, and c-abl values from K562 cells, primary PBMNCs, and purified CD34⁺ CML cells 24 hours after electroporation with the respective siRNAs. GL2-siRNA controls (GL2) for each patient were set at 100%. K562 data represent mean ± SD from 4 independent experiments.

ND indicates not done.

Table 2. Effects of anti-bcr-abl siRNAs on colony formation of primary CML cells

Cell sample/siRNA	CFU-GM	BFU-E
CML-PBMNCs		
No. 1		
GL2	7	15
b3a2_1	7	13
No. 2		
GL2	45	64
b3a2_1	43	43
No. 3		
GL2	5	2
b3a2_1	3	1
No. 4		
GL2	23	53
b3a2_1	22	45
CML-CD34⁺ cells		
No. 5		
GL2	30	17
b3a2_1	32	17
No STI571	29	14
1 μM STI571	4	2
No. 6		
GL2	128	72
b3a2_1	88	66

Standard colony assays were performed with PBMNCs (patient samples 1-4) after electroporation with the respective siRNA. Purified CD34⁺ cells from patient sample 5 were initially cultured in standard colony assays, and the samples "No STI571" and "1 μM STI571" were not electroporated. The last 4 lanes show CD34⁺ cells (patient samples 5, 6) first grown in suspension culture for 3 days and then plated into standard colony assays. No viable cells were found after suspension culture in the presence of 1 μM STI571 after 3 days (patient sample 5).

CFU-GM indicates colony-forming unit granulocyte macrophage; and BFU-E, burst-forming unit erythroid.

Our data show gene suppression mediated by siRNA in normal and malignant hematopoietic cells. Specifically, siRNAs induced a specific but transient reduction of bcr-abl mRNA and protein expression, and an inhibition of BCR-ABL-mediated cell proliferation. As expected for mammalian cells,^{2,3,23} we found no evidence for transitive RNAi involving RdRP because anti-bcr-abl siRNAs did not affect c-bcr and c-abl mRNA levels. The markedly different effects of anti-bcr-abl siRNA and STI571 on CML cells in our study may be explained by the transient and nonheritable nature of RNAi in mammalian cells and the protein half-life of BCR-ABL. Alternatively, the inhibition of bcr-abl expression by siRNA or antisense sequences²⁴ compared with blocking BCR-ABL kinase activity by STI571 may induce different phenotypes in cytokine-supplemented cultures of bcr-abl-positive cells.^{22,24} The molecular basis for these differences, and the kinetics of RNAi triggered by exogenous or endogenous expression of siRNAs,^{10,25} should be analyzed to better define the role of RNAi as scientific tools or potential therapeutics in human hematopoietic cells.

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