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ORIGINAL ARTICLE

# Additive antileukemia effects by *GFI1B*- and *BCR–ABL*-specific siRNA in advanced phase chronic myeloid leukemic cells

M Koldehoff[1], JL Zakrzewski[2], DW Beelen[1] and AH Elmaagacli[1]

Previous studies demonstrated selective inhibition of the BCR–ABL (breakpoint cluster region–Abelson murine leukemia oncogene) tyrosine kinase by RNA interference in leukemic cells. In this study, we evaluated the effect of *BCR–ABL* small interfering RNA (siRNA) and *GFI1B* siRNA silencing on chronic myeloid leukemia (CML) cells in myeloid blast crises. The *GFI1B* gene was mapped to chromosome 9 and is, therefore, located downstream of the *BCR–ABL* translocation in CML cells. Co-transfection of *BCR–ABL* siRNA and *GFI1B* siRNA dramatically decreased cell viability and significantly induced apoptosis and inhibited proliferation in K562 cells ($P<0.0001$) and primary advanced phase CML cells ($P<0.0001$) versus controls. Furthermore, combining of *BCR–ABL* siRNA and *GFI1B* siRNA significantly modified the expression of several relevant genes including *Myc*, *MDR1*, *MRP1* and tyrosyl-phosphoproteins in primary CML cells. Our data suggest that silencing of both *BCR–ABL* siRNA and *GFI1B* siRNA is associated with an additive antileukemic effect against K562 cells and primary advanced CML cells, further validating these genes as attractive therapeutic targets.

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**Keywords:** advanced leukemia; *BCR–ABL*; *GFI1B*; K562; small interfering RNA

## INTRODUCTION

Chronic myeloid leukemia (CML) is a relatively well-differentiated myeloproliferative disorder originating from transformed hematopoietic stem cells. It follows a fairly benign course for several years (chronic phase) before transforming into the more aggressive accelerated phase and life-threatening blast crisis (BC). CML is characterized by the Philadelphia chromosome, which results in the expression of the *BCR–ABL* fusion gene, and is derived from the fusion of the cellular breakpoint cluster region (*BCR*) gene and the Abelson murine leukemia oncogene (*ABL*). The expression of these consistent molecular changes has been shown to be necessary and sufficient for the transformed phenotype of CML cells.[1,2] The BCR–ABL protein activates multiple signaling pathways, including the RAS/MEK/extracellular signal-regulated kinase 1 and 2/phosphatidylinositol 3-kinase/Akt, NF-κB and signal transducer and activator of transcription protein pathways.[3,4] BCR–ABL-mediated altered expression of these pathways leads to CML progression. Strategies for targeting cellular structures of neoplastic cells include the use of low-molecular weight pharmacologically active compounds such as tyrosine kinase inhibitors (TKIs), large molecules such as antibodies, and the application of nucleic acid-based inhibitors of gene expression, that is, antisense oligonucleotides, ribozymes, DNAzymes and RNA molecules mediating RNA interference (RNAi).[5] RNAi represents an evolutionarily conserved cellular mechanism that mediates sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA. Small interfering RNAs (siRNA) are the mediators of mRNA degradation in the process of RNAi.[6] We demonstrated that combined transfection of CML cell lines and cells of CML patients with Wilms' tumor gene (*WT1*) siRNA and *BCR–ABL* siRNA leads to increased inhibition of proliferation and induced apoptosis compared with transfection with *BCR–ABL* siRNA or *WT1* siRNA alone.[7] We also showed that silencing by *BCR–ABL* siRNA combined with TKIs such as imatinib or nilotinib may be associated with an additive activity against TKI-sensitive and resistant BCR–ABL$^+$ CML cells, and might represent an alternative approach to overcome TKI resistance due to BCR–ABL mutations.[8]

Growth factor independent-1B is a transcription factor with six $C_2H_2$ zinc fingers and one unique N-terminal SNAG (Snail/Gfi-1) transcriptional repressor domain. The *GFI1B* gene was mapped to chromosome 9q34.13 and is, therefore, located downstream of the *BCR–ABL* translocation in CML cells. Growth factor independent-1B signaling is important for commitment and maturation of hematopoietic cell populations.[9,10] *GFI1B* as target for gene silencing was chosen because it is aberrantly overexpressed in leukemias, including a high overexpression in erythropoietic and megakaryocytic malignancies, advanced CML, and in their corresponding cell lines, especially in K562 cells, a cell line that is derived from CML cells in myeloid BC. Growth factor independent-1B is much less expressed in normal hematopoietic progenitor cells than in leukemic cells, suggesting that silencing of this gene might interfere with proliferation and survival of leukemic cells.[11,12]

We evaluated the antileukemic effects of *BCR–ABL* silencing in advanced or BC leukemic cells, and performed studies to assess if this effect can be augmented by the additional application of siRNA directed against the *GFI1B* gene. In order to elucidate the function of the *GFI1B* gene in advanced leukemia, we profiled gene expression in advanced leukemic cells in response to siRNA treatment.

[1]Department of Bone Marrow Transplantation, West German Cancer Center, Faculty of Medicine, University of Duisburg-Essen, Essen, Germany and [2]Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY, USA. Correspondence: Dr M Koldehoff, Department of Bone Marrow Transplantation, West German Cancer Center, Faculty of Medicine, University of Duisburg-Essen, Hufelandstreet 55, 45122 Essen, Germany.
E-mail: michael.koldehoff@uk-essen.de
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## MATERIALS AND METHODS

### Cell culture

K562 cells, CML in myeloid blast crises, were grown in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum as described[13] and were maintained at 37 °C in a humidified incubator with 5% $CO_2$.

### Cells from patients

Peripheral blood or bone marrow from patients with advanced phase CML was used. Informed consent was obtained before the procedure.

### RNA purification

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

### Quantitative real-time PCR

We quantified *BCR–ABL*, *GFI1B*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *c-Myc*, *MDR1*, *MPR1* and $p21^{CIP1/WAF1}$ mRNA levels by real-time reverse transcription-polymerase chain reaction (RT-PCR) using the Lightcycler (Roche Applied Science, Mannheim, Germany) or the ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems, Darmstadt, Germany) device and software. Primer and hybridization probes for RT-PCR for *BCR–ABL*, *GFI1B*, *MDR1* and *GAPDH* have been previously published.[10,11,14] For $p21^{CIP1/WAF1}$, *c-Myc* and *MPR1* we used the primers and hybridization probes as published earlier.[15–17] Quantification of RNA transcript expression was normalized determining the ratio between expression levels of targets and *GAPDH*.

### siRNA transfection

For *in vitro* transfection with *GFI1B* siRNA, a total of 175 pM *GFI1B* siRNA (purchased from Qiagen, No. AF 081946, sense 5′-CAGCCCUGUCCUUAG CACUDTDT-3′ and antisense 5′-AGUGCUAAGGACAGGGCUGDTDT -3′sequences) was added to treated cells and transfection was performed as previously described.[8,13] Briefly, *in vitro* transfections with siRNA were performed in 24-well plates using the DOTAP liposomal transfection reagent ($1 \times 10^5$ cells per well) (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol. Sequences of siRNA directed against the *BCR–ABL* transcript were published previously by Scherr *et al*.[18] The *BCR–ABL* siRNA was also purchased by Qiagen and transfection with 54 pM *BCR–ABL* siRNA was performed as described for *GFI1B* siRNA. As control, we used two or more non-silencing siRNA (mismatched or scrambled siRNA) from Qiagen.

### Growth inhibition assay

Cells were cultured in 96-well plates at a concentration of 5000 cells per well and left to recover. The number of viable cells was quantitatively estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). MTT (10 µl of 5 mg ml$^{-1}$ solution, Sigma Chemical, Hamburg, Germany) was added to each well of the titration plate and incubated for 4 h at 37 °C. The cells were then solubilized by the addition of dimethyl sulfoxide (40 µl per well) and incubated for 60 min at 37 °C. The absorbance of each well determined in an enzyme-linked immunosorbent assay plate reader using an activation wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of viable cells was determined by comparison with untreated control cells.

### Terminal transferase deoxyuridine triphosphate nick-end labeling assay

Apoptotic cells were determined using the *in situ* cell Death Detection kit from Roche Molecular Diagnostics (Mannheim, Germany) following the manufacturer's instruction. The apoptotic cells (brown staining) were counted under a microscope. The apoptotic index was defined by the percentage of brown (dark) cells among the total number of cells in each sample. Five fields with 100 cells in each were randomly counted for each sample. At least three samples with fifteen single analyses were counted.

### Cell proliferation assay

Cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) incorporation. Twenty-four hours (and up to 48 h) after transfection of siRNA, the treated cells were split into four-well chamber slides and incubated with culture medium containing BrdU for 4 h. BrdU staining was performed using the Roche Kit (Mannheim, Germany) following the manufacturer's instructions. Analysis of proliferation was defined as the percentage of brown stained cells among the total number of cells per sample and performed likewise as the analysis of apoptotic cells.

### Flow cytometry

$1.0–5.0 \times 10^5$ cells were labeled with antibodies for multi-color flow cytometry using fluorescein isothiocyanate, phycoerythrin or PerCP conjugated monoclonal antibodies directed against CD45, CD243 (P-gp), anti-phosphotyrosine protein (p-Tyr, PY99) and 7-AAD. For intracellular anti-phosphotyrosine protein (p-Tyr, PY99) and anti-phospho-Crkl (p-Tyr207) staining, cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.1% Nonidet P40 for 4 min before intracellular staining. Anti-phosphor-Crkl (p-Tyr207) was performed for 30 min at room temperature in the dark. After incubation with the primary antibody, cells were subsequently washed twice with buffer and incubated with PC5-labeled goat anti-rabbit immunoglobulin G secondary antibody. Cells were again washed and resuspended in 500 µl of PBS for flow cytometry analysis. All antibodies were obtained either from Beckmann-Coulter (Krefeld, Germany) or from Santa Cruz Biotechnology (Heidelberg, Germany). Nonspecific binding was corrected with isotype-matched controls. Flow cytometric data were acquired using a four-color Epics XL AF 14075 flow cytometer with Expo 32 ADC software (Coulter).

### Statistics

Values are presented as mean ± s.d. Variations in data between the different groups were tested either by a two-tailed unpaired *t*-test or a Mann–Whitney *U*-test using the SPSS 11.5 program (SPSS Inc., Chicago, IL, USA).

## RESULTS

### *GFI1B* expression in primary CML, K562 cells and hematopoietic progenitor cells

Eighteen bone marrow samples from patients with CML in different stages and two $CD34^+$-enriched hematopoietic progenitor samples from healthy volunteers were evaluated for their *GFI1B* expression measured by real-time RT-PCR and normalized to the housekeeping gene *GAPDH* (quotient of *GFI1B/GAPDH*). High levels of mRNA *GFI1B* were observed in CML patients in chronic phase ($P < 0.01$ compared with $CD34^+$-enriched hematopoietic progenitor controls). At least 35-fold increased *GFI1B* expression was found in CML patients with advanced phase ($P < 0.01$) and 75-fold increased *GFI1B* expression in CML patients in BC ($P < 0.04$) compared with the mean *GFI1B* expression in hematopoietic progenitor cells of healthy volunteers. Consistent with these observations, *GFI1B* expression was also increased in K562 cells. However, no significant differences in *GFI1B* expression were found in CD34-depleted hematopoietic progenitor samples compared with $CD34^+$-enriched hematopoietic progenitor samples from healthy volunteers. *GFI1B* mRNA expression in patients with CML in advanced phase and in patients with CML in BC was strongly increased compared with patients with CML in chronic phase (58.2 ± 13.5%, $P < 0.02$ and 122.9 ± 52.1%, $P < 0.04$ versus 36.7 ± 13.6% in CML, chronic phase), as seen in Table 1.

To analyze the efficiency of RNAi delivery, we evaluated the transfection rate in six samples of primary CML cells and in K562 cells using fluorescently marked non-silencing siRNA. Twenty-four hours after transfection, the number of transfected cells was evaluated using fluorescence microscopy counting $5 \times 100$ cells. We found a mean transfection rate of 45.8 ± 12.8% in primary advanced CML cells and a mean transfection rate of 69.4 ± 5.7% in K562 cells.

### Growth inhibition of K562 cells

We next assessed whether transfection of *BCR–ABL* siRNA, *GFI1B* siRNA or the co-transfection with these two siRNAs affected the viability of K562 cells. Cells were transfected with constant doses (54 pM for BCR–ABL and 175 pM for GFI1B siRNA) of *BCR–ABL* siRNA, *GFI1B* siRNA, scambled siRNA or no siRNA for 48 h, harvested, and analyzed for cell viability by an MTT assay. As shown in Figure 1a, *BCR–ABL* siRNA, *GFI1B* siRNA and the combination of *BCR–ABL* siRNA with *GFI1B* siRNA significantly decreased the cell viability of K562 cells. *BCR–ABL* siRNA or the combination of both siRNAs revealed the strongest effects, reducing the viability of K562 cells by 28.5 to 5.3% (mean) compared with controls (one and/or two scrambled siRNAs). *GFI1B* siRNA reduced the viability of these cells by 49.8% (mean). Mismatched controls did not inhibit cell viability even when we doubled the siRNA dose (data not shown).

To confirm that cell growth is dependent on the timing of siRNA silencing, we next investigated the time course of effects induced by co-transfection of *BCR–ABL* siRNA, *GFI1B* siRNA and scambled siRNA (or no siRNA) in K562 cells. A significant reduction of cell growth to 21.9% ± 4.5 compared with the spontaneous growth rate of viable cells was found 24 h after siRNA transfection ($P < 0.0001$). We furthermore observed a strong inhibition of cell growth to 5.3% ± 1.8 ($P < 0.0001$) compared with the spontaneous cell growth rate after 48 h of co-transfection with these siRNAs. Seventy-two hours after co-transfection of *BCR–ABL* siRNA and *GFI1B* siRNA, no further reduction of cell growth was measured in K562 cells, in fact cell growth increased to 8.6% ± 3.2 compared with the spontaneous cell growth rate, as shown in Figure 1b.

Taken together, these data indicate that *BCR–ABL* siRNA, *GFI1B* siRNA or the co-transfection of both siRNAs inhibits growth of K562 cells.

**Table 1.** *GFI1B* expression in K562, primary CML cells and hematopoietic progenitor cells measured by real-time RT-PCR

| *Disease* | *GFI1B expression (%)* | P-*value compared with healthy volunteers CD34$^+$ cells* |
|---|---|---|
| K562 ($n = 18$) | 218.4 ± 96.7 | < 0.01 |
| CML in first chronic phase ($n = 10$) | 36.7 ± 13.6 | < 0.01 |
| CML in advanced phase ($n = 4$) | 58.2 ± 13.5 | < 0.01 |
| CML in blastic crisis ($n = 4$) | 122.9 ± 52.1 | < 0.04 |
| *Healthy volunteers* | | |
| CD34$^+$ cells ($n = 2$) | 1.65 ± 0.35 | – |
| CD34$^-$ cells | 0.16 ± 0.01 | NS |

Abbreviations: CML, chronic myeloid leukemia; NS, not-significant; RT-PCR, reverse transcription-polymerase chain reaction. *GFI1B* mRNA expression was measured and shown as mean (with s.d.) normalized to *GAPDH* housekeeping gene expression and compared with gene expression in hematopoietic progenitor cells of healthy volunteers.

### *BCR–ABL* gene expression measured by real-time RT-PCR

We quantified *BCR–ABL* expression in K562 cells in correlation to the housekeeping gene *GAPDH* by real-time RT-PCR (quotient of *BCR–ABL/GAPDH*) and found a significant reduction of *BCR–ABL* mRNA levels to amounts ranging from 135.3 to 62.4% (mean) after transfection with either *GFI1B* siRNA or with *BCR–ABL* siRNA compared with controls, as shown in Table 2. Co-transfection of *BCR–ABL* siRNA and *GFI1B* siRNA accomplished a further reduction of *BCR–ABL* mRNA to 33.2% (mean) compared with controls ($P < 0.0001$). The combination of both *BCR–ABL* siRNA and *GFI1B* siRNA reduced the *BCR–ABL* mRNA level by twofold compared with transfection with *BCR–ABL* siRNA alone ($P < 0.0001$).

### *GFI1B* gene expression measured by real-time RT-PCR

Concordantly with the inhibition of *BCR–ABL* gene expression, we quantified *GFI1B* gene expression in correlation to *GAPDH* by real-time RT-PCR (quotient of *GFI1B/GAPDH*) after transfection of *BCR–ABL* siRNA, *GFI1B* siRNA or the co-transfection of these two siRNAs. Fourty-eight hours after transfection with *GFI1B* siRNA, we observed a significant reduction of *GFI1B* mRNA levels to 32.1% (mean) compared with controls in K562 cells ($P < 0.0001$). We found only a very mild reduction of *GFI1B* mRNA levels to 132.5% (mean) after *BCR–ABL* siRNA transfection. The combination of *BCR–ABL* siRNA and *GFI1B* siRNA strongly reduced the *GFI1B* mRNA level to 25.5% (mean) compared with controls ($P < 0.0001$) (Table 2). Comparing transfection with *GFI1B* siRNA alone with the co-transfection of *BCR–ABL* siRNA and *GFI1B* siRNA, we found a mild reduction of *GFI1B* mRNA levels after transfection with both siRNAs.

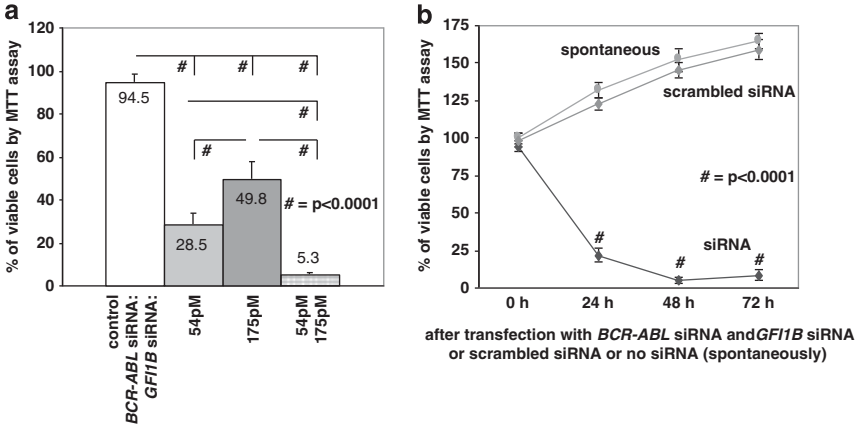


**Figure 1.** Growth rate of K562 cells after transfection with *BCR–ABL* siRNA, *GFI1B* siRNA and cotreatment with both siRNAs as determined by the MTT assay. (**a**) Forty-eight hours after siRNA transfection, the number of viable K562 cells decreased significantly ($P < 0.0001$). (**b**) Kinetics of growth inhibition of K562 cells following application of 175 pM *GFI1B* siRNA and 54 pM *BCR–ABL* siRNA or two scrambled siRNAs and/or without siRNAs is shown. Mean and s.d. are presented. The percentage of viable cells was determined by comparison with untreated or scrambled siRNA-transfected cells. *BCR–ABL* siRNA, breakpoint cluster region–Abelson murine leukemia oncogene small interfering RNA.

Additive effects of *BCR–ABL* siRNA and *GFI1B* siRNA on induction of apoptosis and inhibition of proliferation

As expected, we saw that transfection with *BCR–ABL* siRNA alone or *GFI1B* siRNA alone inhibited the proliferation rate of K562 cells compared with controls as shown in Figure 2a ($P<0.0001$ for 54 pM *BCR–ABL* siRNA and $P<0.0001$ for 175 nM *GFI1B* siRNA versus controls). Co-transfection with *BCR–ABL* siRNA and *GFI1B* siRNA resulted in an exaggerated decrease of the proliferation rate to 9.9% ± 1.7 compared with controls (controls were set up to 100%). The inhibitory efficiency of the combination of *BCR–ABL* siRNA and *GFI1B* siRNA was three times higher than the one of *BCR–ABL* siRNA alone and fivefold increased compared with *GFI1B* siRNA alone ($P<0.0001$ for *BCR–ABL* siRNA and *GFI1B* siRNA versus *BCR–ABL* siRNA or *GFI1B* siRNA alone). Moreover, we observed an additive effect on induction of apoptosis by co-transfection of *BCR–ABL* siRNA and *GFI1B* siRNA in K562 cells. The rate of induced apoptosis increased from 1 ± 0.1 (controls set to 1.0) to 3.3 ± 0.2, whereas the use of either *BCR–ABL* siRNA alone or *GFI1B* siRNA alone was again less effective (*BCR–ABL* siRNA alone 1.8 ± 0.2 and *GFI1B* siRNA alone 1.5 ± 0.2), as shown in Figure 2b ($P<0.0001$ for *BCR–ABL* siRNA + *GFI1B* siRNA versus *BCR–ABL* siRNA or *GFI1B* siRNA alone).

Taken together, these data show that *BCR–ABL* siRNA, *GFI1B* siRNA or the co-transfection of both of these siRNAs induced apoptosis and inhibited proliferation in K562 cells.

**Table 2.** *BCR–ABL* and *GFI1B* genes expression determined by real-time RT-PCR 48 h after siRNA transfection in K562 cells

| *Treatment in K562 cells* | *BCR–ABL gene expression in % after transfection* | P-*value* | *GFI1B gene expression in % after transfection* | P-*value* |
|---|---|---|---|---|
| Control (mock) | 162 ± 31 | | 142 ± 52 | |
| *BCR–ABL* siRNA (54 pM) | 62 ± 9* | <0.0001 | 133 ± 27* | |
| *GFI1B* siRNA (175 pM) | *135 ± 24 | <0.05 | 32 ± 21 | <0.0001 |
| *BCR–ABL* + *GFI1B* siRNA | 33 ± 8 | <0.0001 | 26 ± 26 | <0.0001 |

Abbreviations: *BCR–ABL*, breakpoint cluster region–Abelson murine leukemia oncogene; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA. *BCR–ABL* gene and/or *GFI1B* gene expression were measured up to eightfold by real-time RT-PCR and results are shown as mean + s.d. normalized to *GAPDH*. *$P<0.0001$.

Additive effects of co-transfection with *BCR–ABL* siRNA and *GFI1B* siRNA in primary CML cells

In order to verify the above-described antileukemic effects, we assessed *BCR–ABL* siRNA and *GFI1B* siRNA transfection in primary cells obtained from four patients with BC of CML (patients with Y253F; F317L; and E255K mutation, and one patient with TKI resistant CML). We found a reduction of *BCR–ABL* mRNA and *GFI1B* mRNA amounts compared with untreated controls. The amount of *BCR–ABL* mRNA was significantly reduced to 20.7% (mean) 48 h after *BCR–ABL* siRNA transfection and to 18.5% (mean) 48 h after co-transfection with *BCR–ABL* and *GFI1B* siRNA, as shown in Figure 3. We observed an additive effect on reduction of *GFI1B* mRNA by co-transfection with *BCR–ABL* siRNA and *GFI1B* siRNA in primary CML cells. The *GFI1B* mRNA level decreased to 57.7% (mean) after *GFI1B* siRNA transfection, whereas the use of cotreatment with *BCR–ABL* siRNA and *GFI1B* siRNA was more effective in inhibiting *GFI1B* mRNA to 54.2% (mean) compared with untreated controls.

Taken together, these data show that co-transfection of *BCR–ABL* and *GFI1B* siRNA efficiently inhibits gene expression in advanced CML cells.

Confirmation of relevant signaling genes and variant protein levels in primary CML cells

In order to investigate the molecular effects on CML cells, we analyzed advanced CML cells with or without siRNA silencing by oligonucleotide microarray technology. After statistical analysis, relevant genes including transcription factors and leukemia-associated genes were chosen for conformation by RT-PCR (data not shown). In this series of experiments, we analyzed *MDR1*, *MPR1*, *c-Myc* and $p21^{CIP1/WAF1}$ in primary CML cells by RT-PCR. We found strongly decreased levels of *MDR1*-, *MPR1*- and *c-Myc* gene expression after co-transfection with *BCR–ABL* siRNA and *GFI1B* siRNA. Levels were decreased to up to 70% compared with

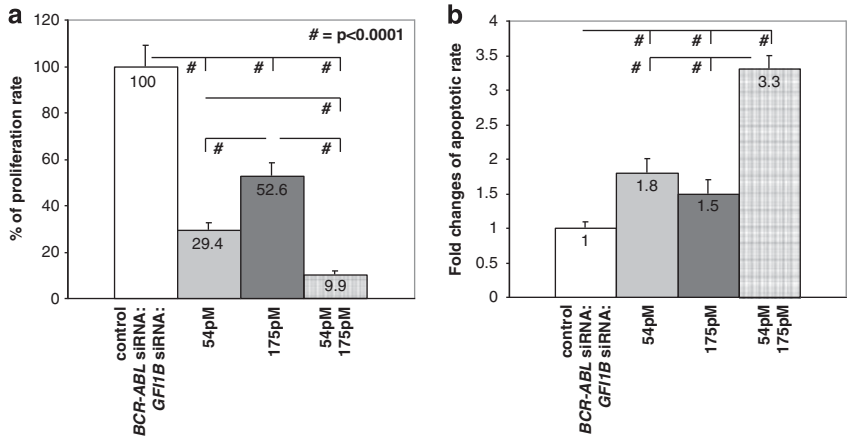


**Figure 2.** (**a**) Proliferation rate of K562 cells measured by 5-bromo-2-deoxyuridine incorporation 48 h after transfection with *GFI1B* siRNA, *BCR–ABL* siRNA and cotreatment with both siRNAs. After transfection with each siRNA, the proliferation rate of K562 cells decreased significantly ($P<0.0001$). Mean and s.d. are presented. (**b**) Apoptosis rate of K562 cells measured by terminal transferase deoxyuridine triphosphate nick-end labeling assay 48 h after transfection with *GFI1B* siRNA, *BCR–ABL* siRNA and cotreatment with both siRNAs. After transfection with each siRNA, the apoptotic rate of K562 cells increased significantly ($P<0.0001$). Mean and s.d. are presented. *BCR–ABL*, breakpoint cluster region–Abelson murine leukemia oncogene; siRNA, small interfering RNA.

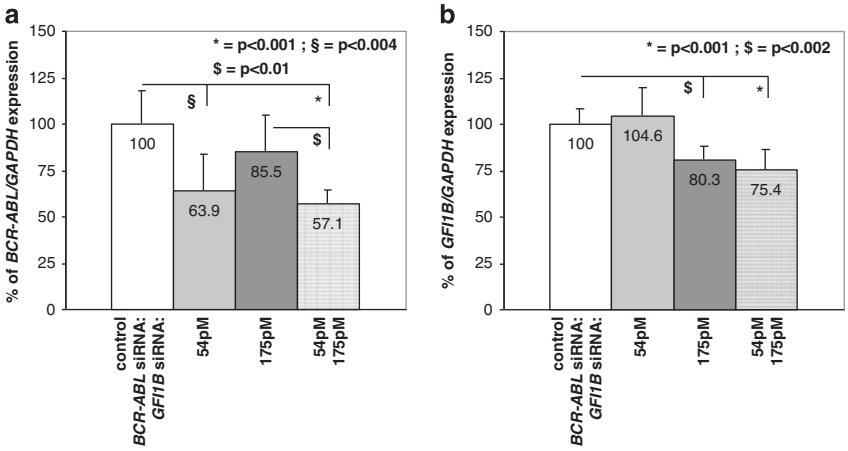


**Figure 3.** *BCR–ABL* and *GFI1B* gene expression determined by real-time RT-PCR 48 h after siRNA transfection of primary advanced CML cells. (**a**) Forty-eight hours after silencing with both siRNAs in primary cells, the *BCR–ABL* expression decreased significantly ($P<0.001$). (**b**) Forty-eight hours after silencing with both siRNAs in primary cells, the *GFI1B* expression decreased significantly ($P<0.001$). Mean and s.d. are presented. Gene expression normalized to GAPDH by real-time RT-PCR. Control was set to 100%. *BCR–ABL*, breakpoint cluster region–Abelson murine leukemia oncogene; CML, chronic myeloid leukemia; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA.

**Table 3.** Analysis of target gene and protein expression 48 h after siRNA transfection of primary advanced CML cells

| *Gene expression* | *Control (mock)* | *BCR–ABL siRNA (54 pM)* | *GFI1B siRNA (175 pM)* | *BCR–ABL + GFI1B siRNA* |
|---|---|---|---|---|
| *MDR1* | 54.6 ± 12.9 | 25.4 ± 8.1*** | 42.8 ± 12.9 | 17.6 ± 10.8*** |
| *MPR* | 7.6 ± 1.2 | 5.9 ± 1.8* | 6.8 ± 1.5 | 3.0 ± 1.2** |
| *p21$^{CIP1/WAF1}$* | 4.4 ± 0.8 | 4.6 ± 0.7 | 5.3 ± 0.8* | 4.6 ± 0.9 |
| *c-Myc* | 18.3 ± 5.2 | 17.3 ± 4.1 | 22.0 ± 3.2 | 12.7 ± 4.7* |
| *Target expression* | | | | |
| CD243/p-gp | 55.5 ± 0.6 | 40.0 ± 0.9** | 39.1 ± 1.2** | 37.4 ± 2.5** |
| p-Crkl | 76.0 ± 5.1 | 45.8 ± 9.1** | – | 36.7 ± 1.6** |
| p-Tyr | 85.6 ± 2.7 | 46.9 ± 1.4** | – | 52.9 ± 3.7** |

Abbreviations: *BCR–ABL*, breakpoint cluster region–Abelson murine leukemia oncogene; CML, chronic myeloid leukemia; siRNA, small interfering RNA. Genes expression normalized to *GAPDH* by real-time RT-PCR or protein levels by flow cytometry were measured up to fivefold and results are shown as mean + s.d. *P*-values are in ***$P<0.001$; in **$P<0.01$; and in *$P<0.05$, respectively.

controls (controls were set up to 100%), as shown in Table 3. P21$^{CIP1/WAF1}$ gene expression was upregulated after transfection with *GFI1B* siRNA and was not affected by transfection with *BCR–ABL* or cotreatment with *GFI1B* and *BCR–ABL* siRNAs in primary CML cells. To further verify the findings described above, we next performed flow cytometric studies on the effects on phosphotyrosine protein (p-Tyr), phosphor-Crkl (p-Crkl) and CD243 (P-gp) expression to assess signaling pathway proteins. We found a significant reduction or inhibition of p-Tyr expression to 62% and p-Crkl to 48% after co-transfection with *BCR–ABL* siRNA and *GFI1B* siRNA (controls were set to 100%). In addition, we confirmed that similar to MDR1 expression, P-gp protein (CD243) expression is significantly decreased after transfection with *BCR–ABL* siRNA and *GFI1B* siRNA in primary CML cells ($P<0.0001$). Comparing the effects of *BCR–ABL* siRNA and *GFI1B* siRNA transfection on MDR1 expression and P-gp protein level, we discovered that the P-gp protein is less susceptible to siRNA treatment in primary CML cells. Taken together, these data suggest that the combination of *BCR–ABL* siRNA and *GFI1B* siRNA results in enhanced inhibition of signaling gene expression and proteins.

## DISCUSSION

The discovery that siRNA could be delivered effectively to mammalian cells indicates that it might be feasible to treat leukemia by selective intervention in leukemic cell gene regulation.[16,19] There is an increasing number of *in vitro* and *in vivo* reports on RNAi-mediated silencing of *BCR–ABL* fusion gene.[9,10,20] These constructs induce target-specific cleavage of *BCR–ABL* mRNA without affecting the expression of *c-BCR* or *c-ABL* mRNA.[21] Moreover, the first report on *BCR–ABL* siRNA by Wilda *et al.*[22] showed that *BCR–ABL* silencing was accompanied by strong induction of apoptotic cell death. The rate of induced apoptosis was even as high as that induced by 1 μM imatinib. Other investigators have confirmed these effects of *BCR–ABL* siRNA on CML cells.[23,24] In this study, we examined the effects of siRNA directed against *BCR–ABL* and/or *GFI1B*, a gene downstream of the *BCR–ABL* signaling in CML cells. We found that both *GFI1B* siRNA and *BCR–ABL* siRNA inhibit the growth of K562 cells and primary advanced CML cells in a time-dependent manner (contingent on the transfection efficiency). The response to a single siRNA dose reached a plateau with a maximal two to threefold growth reduction after siRNA transfection. It should be emphasized that the efficiency of siRNA-mediated gene silencing is affected by a combination of factors such as variation in transfection efficiency, siRNA sequences, properties of the target mRNA and others.[18,25] As postulated, we found that coadministration of *GFI1B* siRNA and *BCR–ABL* siRNA resulted in enhanced inhibition of cell growth, *BCR–ABL*- and *GFI1B* gene expression. This may be due to effective modulation by a break-point-specific siRNA or silencing of complex signal transduction pathways controlling proteolysis of potential leukemic effectors in advanced BCR–ABL-transformed cells.[26,27] In addition to regulating gene expression at the transcriptional level, it is increasingly clear that *BCR–ABL* and *GFI1B* are also involved in post-transcriptional regulation via shuttling of heterogeneous nuclear ribonucleoproteins, microRNAs or regulatory RNAs.[28,29] Recently, Schulz *et al.*[30] identified *GFI1B*, using a complementary DNA library screen, as potent negative regulator of Rag (recombination-activating gene). Using the Abelson system, they demonstrated that GFI and growth factor independent-1B

bind directly to a region upstream of Erag, and that the binding of these proteins is followed by changes in the chromatin structure at the Rag locus, whereas indirect inhibition is achieved through repression of the trans-activator FoxO1. Furthermore, we found additive effects of *GFI1B* and *BCR–ABL* RNAi on apoptosis and inhibition of proliferation of treated cells. The proliferation rate decreased by two- to fivefold compared with the use of *GFI1B* siRNA alone ($P<0.0001$). In addition to the inhibition of proliferation, apoptosis was increased threefold after transfection with *GFI1B* and *BCR–ABL* siRNA ($P<0.0001$). This suggests that *GFI1B* and *BCR–ABL* siRNA affects leukemic cells by modulating or transforming genes via different pathways promising additive selective antitumor activity. Recently, Ohba *et al*.[31] demonstrated by microarray analysis of K562 cells that there is crosstalk between siRNA interference and *BCR–ABL* oncogeny, and found that the expression $\sim$250 genes were changed. RNAi of *BCR–ABL* was accompanied by decreased expression of various protooncogenes, growth factors, factors related to kinase activity and factors related directly to cell proliferation. Several factors responsible for the development of apoptosis increased, including signal transducer and activator of transcription-induced signal transducer and activator of transcription inhibitors and apoptosis-related RNA-binding protein. The use of several siRNAs to induce additive effects toward target cells has already been described in other systems.[9,10] In experiments focused on cell cycle analysis, regulatory RNA and apoptotic responses induced by RNAi with cotreatment of *GFI1B* siRNA and *BCR–ABL* siRNA seemed to be fluctuating. There are several pharmacological mechanisms by which CML cells develop resistance to TKIs or other treatments, such as increased drug-efflux-related surface molecules including MDR1, or aberrant regulation of signal transduction.[32] Of note, our real-time RT-PCR data show a highly significant reduction of *MDR1*, *MPR1* and *c-Myc* expression of $>$40–70% after co-transfection with *GFI1B* siRNA and *BCR–ABL* siRNA in advanced phase CML cells. Myc overexpression during CML progression is consistent in CML cells during BC, where *c-Myc* promotes genomic instability and differentiation arrest and correlates with poor responses to imatinib. It is also conceivable that clones with high *c-Myc* expression are selected during CML progression and *c-Myc* high cells are more prone to progress to blast phase.[33] In addition to impaired drug-efflux-related surface molecules, the tyrosyl-phosphoprotein proteins p-Crkl and p-Tyr were significantly reduced up to 40% after co-transfection with both siRNAs. This strongly suggests that dual inhibition of *BCR–ABL* by RNAi additively enhances cytotoxicity and apoptosis in advanced leukemia cells.

This study demonstrates that both *BCR–ABL* siRNA and *GFI1B* siRNA are able to induce apoptosis and inhibition of proliferation of leukemic cells. Moreover, combining *GFI1B*-specific siRNA with *BCR–ABL*-specific siRNA has additive beneficial effects and may reduce overexpression of *c-Myc* and *MDR1* genes. We conclude that siRNA-based strategies, including the use of *GFI1B*-specific siRNA should have true potential for the development of innovative treatment options for patients with advanced leukemia.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

MK designed, performed and analyzed research and wrote the manuscript. JLZ contributed in writing of the paper. DWB and AHE participated in coordination of the study, and funded the study.

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