Suppression of bcr-abl synthesis by siRNAs or tyrosine kinase activity by Glivec alters different oncogenes, apoptotic/antiapoptotic genes and cell proliferation factors (microarray study)

Zhivko Zhelev[a], Rumiana Bakalova[a,*], Hideki Ohba[a], Ashraf Ewis[a], Mitsuru Ishikawa[a], Yasuo Shinohara[a,b], Yoshinobu Baba[a,b]

[a] *Single-Molecule Bioanalysis Laboratory, National Institute for Advanced Industrial Science and Technology, AIST-Shikoku, 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan*
[b] *Faculty of Pharmaceutical Sciences, Tokushima University, Tokushima, Japan*

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Abstract Short 21-mer double-stranded/small-interfering RNAs (ds/siRNAs) were designed to target bcr-abl mRNA in chronic myelogenous leukemia. The ds/siRNAs were transfected into bcr-abl-positive K-562 (derived from blast crisis chronic myelogenous leukemia), using lipofectamine. Penetrating of ds/siRNAs into the cells was detected by fluorescent confocal microscopy, using fluorescein-labeled ds/siRNAs. The cells were treated with mix of three siRNA sequences (3×60 nM) during 6 days with three repetitive transfections. The siRNA-treatment was accompanied with significant reduction of bcr-abl mRNA, p210, protein tyrosine kinase activity and cell proliferation index. Treatment of cells with Glivec (during 8 days with four repetitive doses, 180 nM single dose) resulted in analogous reduction of cell proliferation activity, stronger suppression of protein tyrosine kinase activity, and very low reduction of p210. siRNA-mix and Glivec did not affect significantly the viability of normal lymphocytes. Microarray analysis of siRNA- and Glivec-treated K-562 cells demonstrated that both pathways of bcr-abl suppression were accompanied with overexpression and suppression of many different oncogenes, apoptotic/antiapoptotic and cell proliferation factors. The following genes of interest were found to decrease in relatively equal degree in both siRNA- and Glivec-treated cells: Bcd orf1 and orf2 proto-oncogene, chromatin-specific transcription elongation factor FACT 140-kDa subunit mRNA, gene encoding splicing factor SF1, and mRNA for Tec protein tyrosine kinase. siRNA-mix and Glivec provoked overexpression of the following common genes: c-jun proto-oncogene, protein kinase C-α, pvt-1 oncogene homologue (myc activator), interleukin-6, 1-8D gene from interferon-inducible gene family, tumor necrosis factor receptor superfamily (10b), and STAT-induced STAT inhibitor.

1. Introduction

The bcr-abl oncogene is a result of a reciprocal translocation between the long arms of chromosome 9 and 22, encoding a cytoplasmic fusion oncoprotein with tyrosine kinase activity that inhibits apoptosis and triggers malignant transformation. Bcr-abl fusion is a single causative abnormality in chronic myelogenous leukemia (CML), at least in chronic phase, making it a unique model for development of molecular targets for disease control [1–3]. Gene or protein targeting of the chimeric fusion is a promising way to eliminate the bcr-abl-positive cells specifically, without any influence on the viability of normal cells. Increasing knowledge on bcr-abl resulted in a design of several novel therapeutic approaches, including highly specific tyrosine kinase inhibitors (as Glivec and pyrido[2,3-d]pyrimidine derivative PD180970), single-stranded antisense oligo-RNA and oligo-DNA substances, and immunomodulation [1–4]. Recently, it has been reported that short double-stranded/small-interfering RNA molecules (ds/siRNA), targeting to bcr-abl abnormality, are also promising tool for control of the chimeric fusion protein expression [5–7].

It has been found that the inhibition of bcr-abl synthesis by antisense oligo-DNAs or conventional drugs is accompanied with cross-talk with other oncogenes (e.g., telomerase) and telomere-associated proteins (e.g., tankyrase, TRF1 and Tin2) [8,9]. The overexpression of antiapoptotic and other cell proliferation factors, together with point-mutations in abl-domain [10,11], can be responsible for development of resistance to the conventional anti-bcr-abl drugs after their long-term application.

The discovery of RNA interference (RNAi) mechanism in mammalian cells in 2001 [12,13] enhanced the expectations to decide the problems of gene therapy of bcr-abl abnormality. The efforts were directed to the application of this natural phenomenon for a specific influence of oncogene and oncoprotein expression.

RNAi is conserved in a diverse variety of simpler organisms (such as nematodes, plants and *Drosophila*) [14–16]. RNAi is mediated by siRNAs that are produced from long dsRNAs of exogenous or endogenous origin by an endonuclease of the ribonuclease-III type, called Dicer. The resulting siRNAs are about 21–23 nucleotides long and these short fragments serve as guide sequences to induce target-specific mRNA cleavage by the RNA-induced silencing complex [17–19]. This mechanism is extremely potent and requires only a few dsRNA molecules per cell to silence homologous gene mRNA expression.

* Corresponding author. Fax: +81-87-8694113.
E-mail address: r.bakalova-zheleva@aist.go.jp (R. Bakalova).

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It has been widely accepted that siRNAs are useful tool in gene knockdown experiments and ultimately for therapeutic purposes because siRNA-mediated transcriptional silencing is highly gene-specific and without secondary effects. However, caution is needed because of the findings that some siRNAs induce interferon-stimulated genes in mammalian cells in vitro [20]. Moreover, it is too early to say how great is the promise of RNAi because of many queries to the estimation of real efficacy of siRNAs, as their extremely difficult intracellular delivery without transfectants, transient nature of RNAi, side-effects during long-term treatment, etc.

The present study was designed to compare the effects of two independent pathways for bcr-abl suppression (siRNA-mediated inhibition of bcr-abl protein synthesis and Glivec-mediated inhibition of tyrosine kinase activity of already synthesized bcr-abl protein) on the cross-expression of other oncogenes, apoptotic/antiapoptotic genes and cell proliferation factors that can be responsible for restoration of cell immortalization and development of drug resistance.

2. Materials and methods

2.1. siRNAs

Twenty one nucleotide ds/siRNAs were chemically synthesized (Genenet Co. Ltd., Fukuoka, Japan). They were directed to human bcr-abl fusion peptide (major breakpoint, abl gene). The sense cDNA sequence was in exon 548···843 (number 3, abl gene) – for siRNA-1 and siRNA-3; and in exon 374···547 (number 2, abl gene) – for siRNA-2 (GenBank Accession No. AJ131466.1 and X16416.1). The sense mRNA/cDNA target sequences and antisense siRNA sequences are shown in Fig. 1A. The following mismatch ds/siRNA (non-sense) was used as a negative control: siRNA-4 – r(GUUUGUCAGAGU CGGACAG)d(TT)/r(CUGUCCGACUCUGACAAAC)d(TT).

2.2. Cells and siRNA transfection protocol

Bcr-abl positive K-562 cells (derived from CML patients in blast crisis) were a generous gift of Dr. J. Minowada (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan). The cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 37 °C with 5% CO_2. The cells used for transfection were about 80% confluenced. The cells used for assay were in a logarithmic phase.

Normal lymphocytes were purified from heparinized peripheral blood obtained from normal adults (aged 40–42 years) by Lymphosepar I.

All cells were sedimented by centrifugation (1000 rpm, 10 min) and washed three times by PBS(−) before experiments.

Transfection conditions: collection of cells by centrifugation, resuspendation of cells in a new medium to 8×10^5 cells/ml, transfection of siRNAs (mix of the three sequences – 3×60 nM) using lipofectamine (6 h incubation in RPMI-1640 medium in the presence of 10 U/ml RNase inhibitor (Roche)), replacement of cells in a new medium (4×10^5 cells/ml) and cultivation in a humidified atmosphere. The conditions for preparation of ds/siRNA–lipofectamine complexes are described in Lipofectamine'2000 protocol (Invitrogen). Transfection efficacy was analyzed using lamin A/C system (RNAi Started kit, Qiagen). Three repetitive transfections were applied as shown in Fig. 1C.

The half-life of ds/siRNAs in RPMI-1640 medium during transfection was analyzed by gel electrophoresis (using UV-detection) and HPLC (using fluorescein-labeled ds/siRNA and fluorimetric detection).

2.3. Confocal microscopy – ds/siRNAs cellular uptake

Cells (2×10^5 cells/ml) were transfected with fluorescein-labeled ds/siRNA (single dose 180 nM, Qiagene) using lipofectamine as described above. Six hours transient transfection was applied and transfected cells were cultured in RPMI-1640 medium. At different times of post-transfection (1, 2, 4, 6 and 8 h), aliquots of the cells were sedimented by centrifugation ($1000 \times g$/10 min), washed twice with PBS (Ca^{2+} and Mg^{2+} free) to eliminate free fluorescent ds/siRNA molecules outside the cells, and the FITC-labeled ds/siRNA, incorporated into the living cells (green light), were detected by fluorescent confocal microscopy. The samples were analyzed by Olympus IX70 microscope.

2.4. Treatment with Glivec

Glivec was kindly provided by Novartis (Novartis, Switzerland). It was applied to the cells in a concentration of 180 nM every two days (the protocol is similar to that in Fig. 1C) during 8 days to obtain analogous to siRNA-protocol reduction of cell proliferation.

2.5. mRNA isolation

mRNA was isolated from ds/siRNA- or Glivec-treated and -untreated cells, using QuickPrep mRNA Purification kit (Amersham Pharmacia), as described in the manufacturer's protocol. Briefly, total RNA was extracted in a buffered solution containing a high concentration of GTC and *N*-lauroyl sarcosine, which ensured the rapid inactivation of endogenous RNase and the complete dissociation of cellular components from the mRNA. The extracts were then diluted threefold with elution buffer (10 mM Tris–HCl, pH 7.5 and 1 mM EDTA) to reduce the GTC concentration to a level enough to allow efficient hydrogen-bonding between poly(A) tracts on mRNA molecules and oligo(dT) attached to cellulose, but high enough to maintain complete inhibition of RNases. Threefold dilution also caused precipitation of some proteins, which were removed by centrifugation ($12\,000 \times g$ for 10 min). The supernatant was poured into an oligo(dT)-cellulose spun column and polyadenylated fraction was allowed to bind over a short period of time with gentle mixing (10 min). The column was subjected to a low-speed centrifugation ($350 \times g$ for 2 min) and the liquid, containing the non-bound material, was decanted. The matrix was batch-washed sequentially with high-salt (10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.5 M NaCl) and then low-salt buffer. Finally, the sample was eluted from the matrix prewarmed at 65 °C with elution buffer (10 mM Tris–HCl, pH 7.5 and 1 mM EDTA). Precipitation of mRNA from eluate (0.5 ml) was carried out by potassium acetate solution (50 μl–5 M, pH 5.0), glycogen solution (10 μl–20 mg/ml in DEPC-treated water) and 95% ethanol (1 ml) for 30 min at −20 °C. The precipitated mRNA was resuspended in DEPC-treated water and its concentration was determined spectrophotometrically at 280 nm. The mRNA isolated was essentially free of DNA and protein contamination. Aliquots of mRNA in equal concentration were applied for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

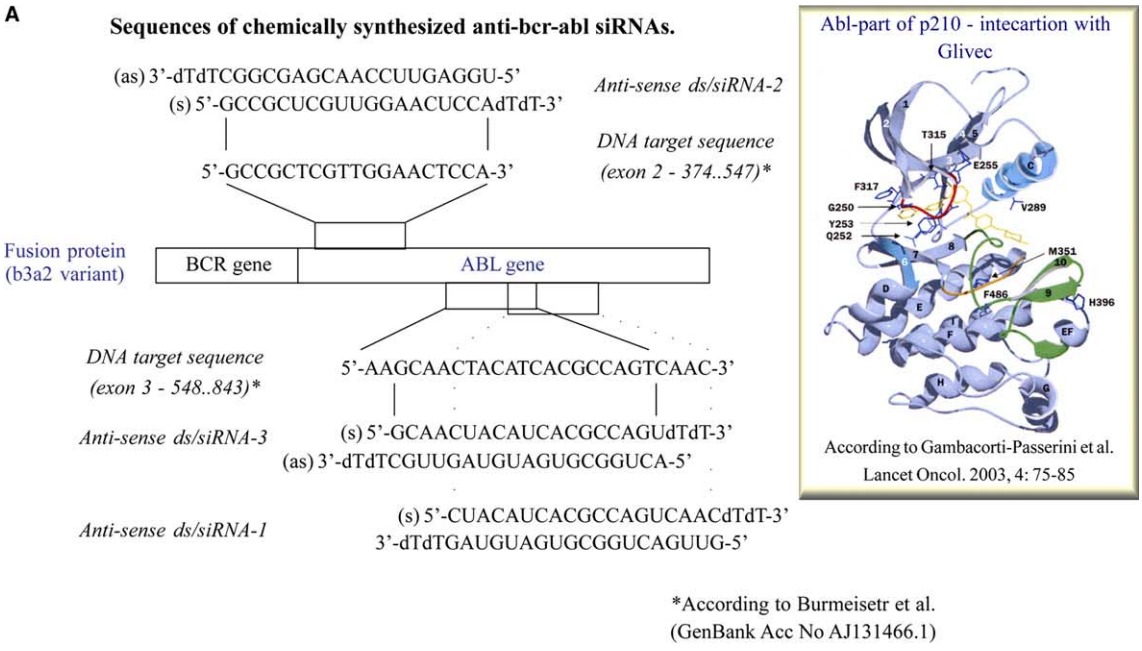
2.6. RT-PCR

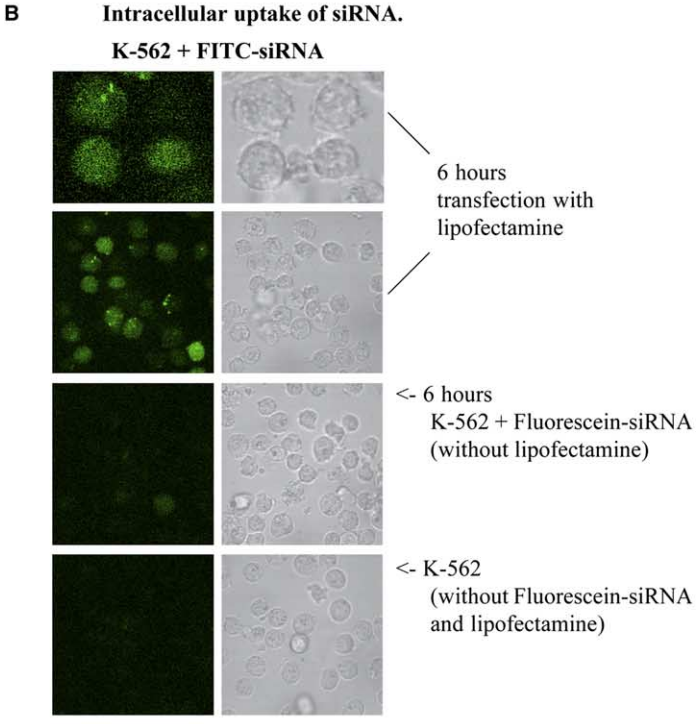
RT-PCR was performed by OneStep RT-PCR kit (Qiagene) in accordance to the manufacturer's instructions. The following gene-specific primers were used: abl-FP (24-mer), 5′-CTC ATA TCA ACC CGA GTG TCT CTT-3′; abl-RP (24-mer), 5′-TGC TAC CTC TGC ACT ATG TCC ATG-3′; bcr-abl fusion-FP (26-mer), 5′-GAA GAA GTG TTT CAG AAG CTT CTC CC-3′; bcr-abl fusion-RP (25-mer), 5′-GAC CCG GAG CTT TTC ACC TTT AGT T-3′. The reverse-transcription reaction was carried out at 50 °C for 30 min, followed by HotStar Taq DNA polymerase activation by heating at 95 °C for 15 min. Three-step cycling was performed: denaturation – 1 min at 94 °C, annealing – 1 min at 50–68 °C, and extension – 1 min at 72 °C. The number of cycles was 40, followed by final extension at 72 °C for 10 min. cDNA (RT-PCR product) was dissolved in loading buffer and was analyzed by electrophoresis on 1% agarose precast gel with ethidium bromide (100 V, 30–40 min, at room temperature, RT ~ 22 °C).

To ensure that there are no artifacts in our RT-PCR procedure, we analyzed also the expression levels of housekeeper β-actin mRNA in each sample as endogenous reference, using commercially available primers (Qnet).

2.7. Western blot analysis of p210 bcr-abl protein

The cells were treated with ds/siRNAs or Glivec as described above. Aliquots of 4×10^5 cells were lysed in TeloChaser buffer (containing protease and phosphatase inhibitor cocktail, Toyobo Co. Ltd.) for 30 min at 4 °C, centrifuged at $12\,000 \times g$/20 min/4 °C and the supernatant was dissolved 1:1 in 2× Laemmli sample buffer (1.1 M Tris–HCl, pH 6.0, 3.3% SDS, 22% glycerol, 10% β-mercaptoethanol and 0.001% bromophenol blue). Samples (in equal protein concentration) were heated at 95 °C for 10 min and were applied to 5% stacking, 4–12% resolving SDS–polyacrylamide gel. Electrophoresis was





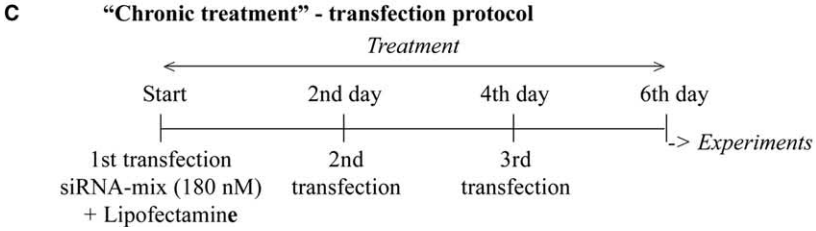


Fig. 1. (A) Sequences of chemically synthesized anti-bcr-abl ds/siRNAs and their sense mRNA/cDNA target sequences. TT indicates the deoxythymidine dimer as 3′ overhang. (B) Intracellular uptake of ds/siRNAs. Cells (2×10^5 cells/ml) was transfected during 6 h with fluorescein-labeled ds/siRNA using lipofectamine as described in Section 2. The cells were sedimented by centrifugation, washed twice with PBS (Ca^{2+} and Mg^{2+} free), and the fluorescein-labeled ds/siRNA, incorporated into the living cells (green light), were detected by fluorescent confocal microscope. (C) Scheme of "chronic treatment" of cells with ds/siRNA-mix or ds/siRNA-non-sense. ds/siRNA-mix consisted of three ds/siRNAs (3×60 nM).

carried out in two steps: at 80 V for 15 min and 120 V for 2 h at RT. BioRad Kaleidoskop protein standards were also applied for comparison. After electrophoresis, the separated protein fractions were transferred to a Hybond-P PVDF membrane (Amersham Bioscience), using XCell II Blot Module (Novex). The transfer was carried out at 35 V for 18 h at 4 °C. The membranes were cut at 45 kDa (for β-actin) and 210 kDa (for bcr-abl) molecular weight levels. A double antibody procedure was used to detect the proteins. The membranes were incubated under agitation for 1 h at RT in a blocking solution (PBS, containing 5% dry skimed milk and 0.1% Tween 20), then at RT for 1 h in anti-c-abl (rabbit, Sigma; 1:100 dilution for bcr-abl detection) or anti-β-actin monoclonal antibodies (mouse, Calbiochem; 1:20 000). The antibody solution was removed and the membranes were washed three times with PBS, containing 0.1% Tween 20. The membranes were then incubated (1 h at RT under agitation) with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, 1:5000) for anti-c-abl and goat anti-mouse IgM (Sigma, 1:2000) for anti-β-actin antibody. Chemiluminescence was detected immediately using ECL Advance Western Blotting Detection Kit (Amersham Bioscience).

2.8. Flow cytometry

Anti-c-abl antibody (rabbit, Sigma) was labeled by ZenonAlexa Fluor-488 Rabbit IgG Labeling Kit (Molecular Probes). Permeabilization of cells (treated or non-treated with ds/siRNA or Glivec) for Fluor-488-conjugated anti-c-abl antibody was carried out by IntraPrep Permeabilization Reagent (Immunotech). The antibody–antigene interaction was detected by flow cytometry (Beckman Coulter-Epics XL). Data were collected and analyzed by "XL System II software". No cells were excluded from the analysis and ~5000 cells were counted. The results were presented as a dot plot of Fluor-488 fluorescence (side scatter, SS – *y*-axis in 0–128 scale, vs. Fluor-488-conjugated antibody, Fluor-488 – *x*-axis in 1–1000 scale) with quadrant markers drawn to distinguish the cells, containing different levels of antibody–antigene complexes.

2.9. Tyrosine kinase activity test

The cells (treated or non-treated with ds/siRNA or Glivec) were sedimented by centrifugation ($1000 \times g$/10 min), washed twice with PBS (Ca^{2+} and Mg^{2+} free) and re-suspended in lysis buffer (TeloChaser, Toyobo Co., Ltd.). The lysis was carried out at 4 °C for 30 min in the presence of orthovanadate. The cytosolic fraction, containing bcr-abl, was obtained after centrifugation at $12\,000 \times g$/10 min, 4 °C. Bcr-abl oncoprotein was isolated together with c-abl (p150) by immunoprecipitation, using anti-c-abl antibody (Sigma, 3 μg/ml) and protein A/G agarose beads. The tyrosine kinase activity of immunoprecipitated enzymes was analyzed, using CHEMICON's PTK Assay kit (with slight modifications). In briefly, a synthetic biotinylated poly[Glu:Tyr], 4:1 (2.5 μg/ml), containing multiple tyrosine residues, was used as a PTK substrate. The PTK reaction was started in cell lysate by 1 mM ATP/10 mM $MgCl_2$ and continued 60 min at 37 °C. After quenching the enzyme reaction with an inhibitor (100 mM EDTA), both the phosphorylated and dephosphorylated substrates were immobilized to the streptavidin-coated 96-well plates. The fraction of phosphorylated substrate was visualized spectrophotometrically at 450 nm, using HRP-conjugated mouse anti-phosphotyrosine antibody (clone PY20) and an ensuing chromagenic substrate reaction. The quantity of phosphate, incorporated into the tyrosine kinase substrate, was determined utilizing the phosphopeptide standard curve.

2.10. Cell proliferation test

Cells (2×10^5 cells/ml) were cultured in the presence or in the absence of ds/siRNAs or Glivec, as described above. The cell growth was detected spectrophotometrically, using CellTiter AQ Proliferation Assay Kit (Promega). The method is based on the detection of the number of viable cells in proliferation. A tetrazolium compound (MTS) is bioreduced by cells into a formazan product in the presence of an electron coupling reagent (phenazine methosulfatef), based on the method of Mosmann [21]. The absorbance of formazan product at 490 nm ($OD_{490\ nm}$) was recorded using 96-well plate reader InterMedImmunomini NJ-2300 (InterMed, Japan).

2.11. Cell viability assay of normal lymphocytes

The viability of siRNA- and Glivec-treated normal lymphocytes was measured by CellTiter-Glo™ luminescent cell viability assay. ATP bioluminescence was used as a marker of cell viability. Briefly, the cells (siRNA/Glivec-treated or untreated) were suspended in the culture medium to a concentration of 5×10^5 cells/ml and then were added in 90 μl aliquots to 96-well plates. CellTiter 96 kit (Promega) was added in aliquots of 90 μl to each patch and incubated with cell suspensions for 1 h, following the procedure recommended by the producer. The luminescence, produced by luciferase-catalyzed luciferin + ATP reaction, was measured by MicroLumat LB96P (Perkin–Elmer). The data were normalized to the control group (consisted of untreated cells). Cell viability of siRNA/Glivec-treated cells was calculated as % of luminescence in comparison with the control.

2.12. Microarray analysis

The RT labeling and hybridization followed the protocol recommended by Agilent Technologies Inc. intended for Agilent microarray analysis. Briefly, a 20-μg aliquot of each mRNA sample was reverse transcribed into a cDNA probe with oligo(dT) primer and labeled nucleotides. The reaction was carried out in a solution containing 50 μM dATP/dGTP/dTTP, 25 μM dCTP, 25 μM cyanine 3 (Cy3)-dCTP (for control non-treated sample) or cyanine 5 (Cy5)-dCTP (for ds/siRNA-mix- or Glivec-treated sample) (Enzo Diagnostics, Inc.) and 400 U MMLV reverse transcriptase at 42 °C for 1 h. The labeling reaction was terminated by incubating the reaction mixture at 70 °C for 10 min. The RNA was then degraded by adding 0.05 μg RNaseIA, followed by incubation at 37 °C for 30 min. Degraded RNA and unincorporated nucleotides were removed using a QIAquick PCR Purification Kit (Qiagen Inc.) according to the instructions of Agilent Technologies Inc. Hybridization was carried out in 22 μl of a hybridization mixture containing cDNA probes, the labeled orientation marker (Deposition Control SP300; Operon Technologies Inc.) and mouse Cot-1 DNA (Invitrogen Corporation) at 65 °C for 17 h. The glass slides were then washed with 0.5× SSC and 0.01% SDS at RT for 5 min, and with 0.06× SSC at room temperature for 2 min. After immediately removing the wash buffer by centrifugation, the glass slides were scanned using GenePix 4000B (Axon Instruments, Inc.) containing a 532 nm laser for Cy3 measurement and a 635 nm laser for Cy5 measurement. Scans were made with a pixel resolution of 5 μm, a laser power of 100%, and a photomultiplier tube voltage of 600 V for the 532 nm laser and 520 V for the 635 nm laser.

To eliminate the effect of lipofectamine on the gene profile of K-562 cells, in siRNA protocol control cells consist of cells treated with lipofectamine only on the scheme, shown in Fig. 1C.

Normalization and analysis of microarray data. Sixteen-bit TIFF images produced by the Axon scanner were analyzed using the GenePixPro 3.0 (Axon Instruments, Inc.) software package. After obtaining Cy3 and Cy5 grayscale images, each pseudo-color image was overlaid, and all spots in the ratio image were defined by accessing the gene list file that described the location of each gene on the microarray. The average of the signal intensity was subtracted from the median of background intensity and outputted with the UniGene and GenBank descriptors to a Microsoft Excel data spreadsheet. Relative expression levels were calculated by global normalization between two samples using all detected genes, after the exclusion of spots annotated as "Agilent QC", "Agilent Blank", and "Buffer".

2.13. Statistical analysis

One-way analysis of variance was employed, followed by Bonferroni's test for truly significant differences. Statistical significance was defined at $P < 0.05$. The statistical procedures were performed with GraphPad InStat software, version 2.04, USA. Data are expressed as mean ± S.D.

3. Results and discussion

The sequences of the originally designed and chemically synthesized ds/siRNAs are shown in Fig. 1A. The DNA target sequences were localized in the abl-part of bcr-abl major breakpoint of Philadelphia chromosome, which is characterized by a bcr exon 3/abl exon 2 (b3a2) junction (an unique sequence in K-562 cells). The selection of DNA target sequences aimed to avoid the most sensitive for point mutations area in abl-part of bcr-abl gene (around Tyr^{315}), responsible for

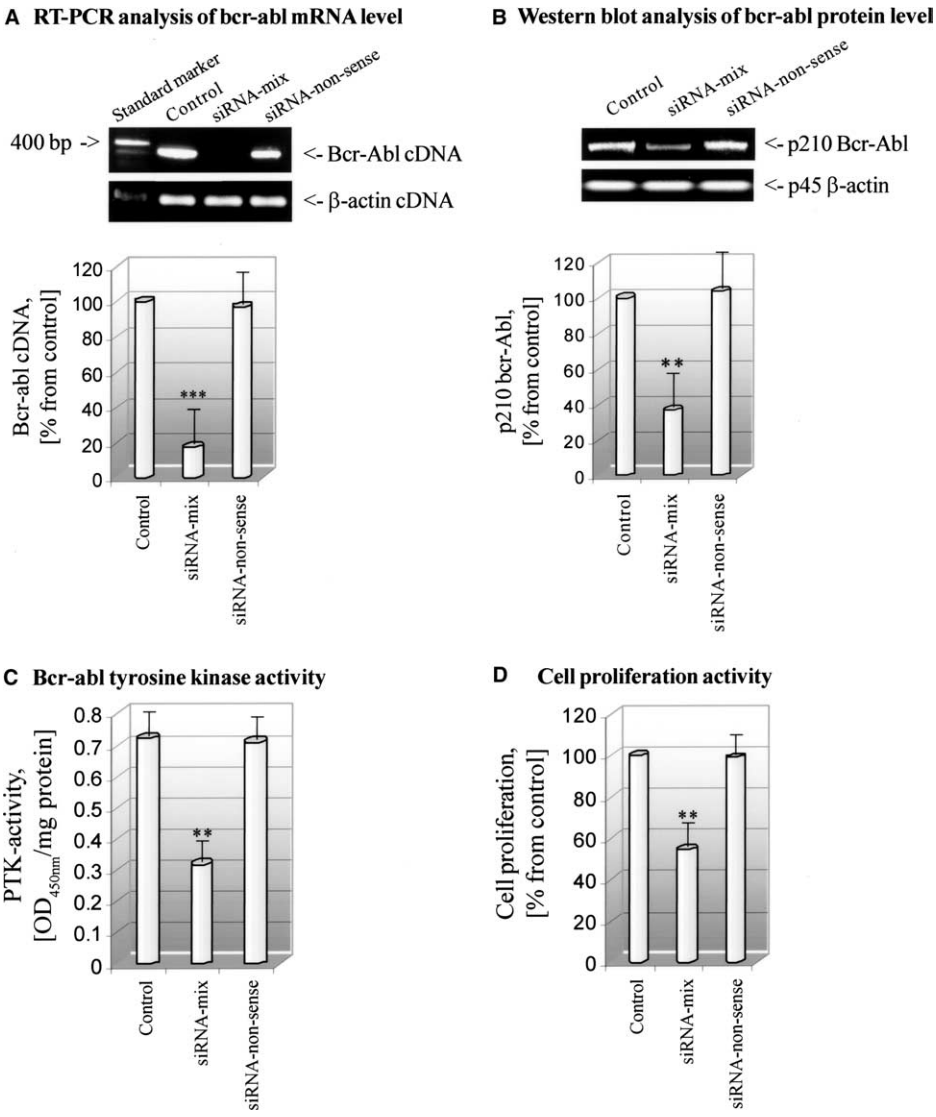


Fig. 2. (A) Effect of ds/siRNA-mix on the bcr-abl mRNA in K-562 cells. The cells were transfected with ds/siRNA-mix (3×60 nM) or ds/siRNA-non-sense (180 nM) as described in Section 2. Cell lysates were obtained after 6 days treatment and mRNA was isolated. The synthesis of bcr-abl cDNA was carried out by RT-PCR and the product was analyzed by TBA-electrophoresis on 1% agarose precast gel with ethidium bromide. Blots from one typical experiment are shown in the figure. In the histograms, the results are expressed as bcr-abl cDNA/β-actin cDNA and were given as a percentage from the control group. The value of bcr-abl cDNA/β-actin cDNA in control (cells treated with lipofectamine only) was considered as 100%. The data are means ± S.D. from six independent experiments. *** $P < 0.001$ vs. control group. (B) Effect of ds/siRNA-mix on the level of bcr-abl oncoprotein in K-562 cells – Western blot analysis. The cells were treated with ds/siRNA-mix (3×60 nM) or ds/siRNA-non-sense (180 nM) and cell lysates were obtained after 6 days treatment as described in Section 2. The samples (in equal protein concentration) were dissolved 1:1 in 2× Laemmli buffer, heated at 95 °C for 10 min and subjected to SDS–polyacrylamide gel electrophoresis (80 V, 15 min; 120 V, 2 h at RT). The separated protein fractions were transferred to a Hybond-P PVDF membrane overnight at 35 V (4 °C). The membranes were cut at 45 kDa (for β-actin) and 210 kDa (for bcr-abl) and were incubated as follows: 1 h at RT in a blocking solution, 1 h in anti-c-abl antibody (rabbit, Sigma, 1:100 for detection of bcr-abl) or in anti-β-actin antibody (mouse, Calbiochem, 1:20 000), 1 h with HRP-conjugated goat anti-rabbit IgG (Sigma, 1:5000) for anti-c-abl or goat anti-mouse IgM (Sigma, 1:2000) for anti-β-actin. Chemiluminescence was detected immediately using ECL Advance Western Blotting Detection kit (Amersham Bioscience). Blots from one typical experiment are shown in the figure. Control blots are representative for cells treated with lipofectamine only. In the histograms, the results are expressed as a percentage from the control group. The value of p210 bcr-abl protein in control (cells treated with lipofectamine only) was considered as 100%. The data are means ± S.D. from five independent experiments. ** $P < 0.01$ vs control group. (C) Effect of ds/siRNA-mix on the protein tyrosine kinase activity in K-562 cells. The cells were treated with ds/siRNA-mix (3×60 nM) or ds/siRNA-non-sense (180 nM) as described in Section 2 and cell lysates were obtained after 6 days treatment. Bcr-abl p210 oncoprotein was isolated together with c-abl p150 by immunoprecipitation, using anti-c-abl antibody (Sigma, 3 μg/ml) and protein A/G agarose beads. The tyrosine kinase activity of immunoprecipitated enzymes was analyzed, using CHEMICON's PTK Assay kit. The fraction of phosphorylated substrate was visualized spectrophotometrically at 450 nm, using HRP-conjugated anti-phosphotyrosine antibody (clone PY20) and ensuing chromagenic substrate reaction. The data are presented as means ± S.D. from five independent experiments. $OD_{450\ nm} = 1.0$ corresponds to 20 ng P-Tyr-peptide substrate. The calibration curve was linear in the range 10–40 ng P-Tyr-peptide substrate. Control groups are representative for cells treated with lipofectamine only. ** $P < 0.01$ vs. control group. (D) Effect of ds/siRNA-mix on the cell proliferation activity of K-562 cells. The cells were cultured in the presence or in the absence of ds/siRNA-mix (3×60 nM) or ds/siRNA-non-sense (180 nM) as described in Section 2. The cell proliferation activity was detected spectrophotometrically, based on the reduction of MTS into a formazan product (absorbance at 490 nm). The data were calculated as a percentage from control (cells, treated with lipofectamine only). The cell proliferating activity of control cells was considered as 100%. The results are presented as means ± S.D. from eight independent experiments. ** $P < 0.01$ vs. control group.

bcr-abl amplification and development of drug-resistance during long-term treatment with conventional antileukemia drugs (as it has been described in the clinical trials with Glivec [10,11]) (Fig. 1A). Thus, the designed ds/siRNAs have a potential to express their activity if they are applied in combination with Glivec during long-term treatment of bcr-abl-positive cells.

Several commercially available liposomal transfection reagents (Lipofectamine 2000 from Invitrogen, Superfect and Lipofect from Qiagen) were tested for their ability to deliver ds/siRNAs into bcr-abl-positive K-562 cells, as well as to preserve ds/siRNAs against degradation in RPMI-1640 medium. K-562 cells were found to be suitable for analysis because they contained a functional RNA interference mechanism to bind to siRNAs and to mediate mRNA degradation, which was confirmed in preliminary experiments by lamin A/C system (RNAi Starter kit, Qiagen, and anti-lamin A/C antibody – Chemicon). Transfection efficacy was also analyzed using lamin A/C system. The preference was given to lipofectamine because of most optimal conditions for comparatively high cellular uptake (transfection efficacy reached approximately 70% in K-562 cells, data not shown) and long half-life of ds/siRNA sequences during transfection. The half-life of fluorescein-labeled ds/siRNA–lipofectamine complexes in cell culture medium was ~10 h, determined by HPLC. The selection of ds/siRNA/lipofectamine ratio meant to avoid also side-effects of lipofectamine on cell viability and proliferation. No significant changes were detected in the viability and proliferation activity of leukemia cells, treated during 6 h with lipofectamine alone or in combination with ds/siRNAs (data are not shown). The cells, treated with lipofectamine only, were used as controls. It was established that only ~20% of ds/siRNAs degradated during 6 h incubation in the medium if the siRNA sequences are in a complex with lipofectamine.

The time-dependent cellular uptake of ds/siRNAs was detected by fluorescent confocal microscopy, using fluorescein-labeled ds/siRNA (Fig. 1B, green light). A comparatively good intracellular uptake of ds/siRNA was detected on the 6th hour of the beginning of transfection. In contrast, ds/siRNA was not detected in the cells without any lipofectamine (no fluorescence was observed in the cells). Based on the preliminary experiments, 6 h transient transfection of ds/siRNAs using lipofectamine was applied and the transfected cells were replaced in a new culture medium.

Bcr-abl protein is considered as "a protein – relatively stable to the antisense attack" and it is difficult to influence its level by single and short-term application of anti-bcr-abl substances. In our previous work, we demonstrated that relatively long-term "chronic" application (6 days and more) of anti-bcr-abl oligo-DNAs markedly reduced the level of bcr-abl proteins and influenced significantly the proliferation activity of K-562 [8,9]. In this context, anti-bcr-abl ds/siRNAs have also a potential to manifest cytotoxicity and to suppress the proliferation during long-term treatment, as it has been assumed by other authors [5,6]. To guarantee enough time for induction of mRNA cleavage as well as for re-synthesis of the target oncoproteins, "chronic treatment" of K-562 cells with ds/siRNAs (mix of the three sequences in equal concentrations – 3×60 nM) was applied. The scheme of "chronic treatment" is shown in Fig. 1C. It includes three repetitive transfections of ds/siRNA-mix (every two days) and the effects on bcr-abl mRNA, p210 and all other parameters were analyzed on the 6th day. The major problem in this experimental protocol was to minimize the side-effects of lipofectamine during repetitive transfections and it was established that the selected scheme overcame this restriction. No significant changes in parameters analyzed were detected in K-562 cells, treated with lipofectamine only as shown in Fig. 1C.

The capacity of ds/siRNA-mix to reduce the amount of bcr-abl mRNA in K-562 cells was estimated by RT-PCR (Fig. 2A). The treatment of cells with ds/siRNA-mix markedly reduced (~82%) the level of bcr-abl mRNA. Similar effect was obtained on the level of p210 bcr-abl oncoprotein, determined by immunoblot analysis (~64% reduction was detected, Fig. 2B). ds/siRNAs-treatment of K-562 cells provoked also a significant decrease of their bcr-abl/c-abl tyrosine kinase activity (~57%, Fig. 2C) and cell proliferation capacity (~50%, Fig. 2D). Mismatch-ds/siRNA did not influence the parameters analyzed, indicating the sequence specificity of ds/siRNA-mix. Obviously, the "chronic treatment" can overcome the transient nature of RNAi and to guarantee comparatively strong suppression of bcr-abl mRNA and oncoprotein expression.

Since the selected ds/siRNAs have a potential to target also c-abl mRNA (Fig. 1A) and to influence the homeostasis of normal cells, we clarified the effect of ds/siRNA-mix (3×60 nM) on the viability of normal lymphocytes after "chronic treatment". It was observed that ds/siRNA-mix did not express cytotoxicity to normal lymphocytes, at least during 6 days treatment on the scheme, as shown in Fig. 1C. This result does not exclude an expression of cytotoxicity of the designed ds/siRNAs to normal cells during long-term treatment and the sequences targeted only to fusion part of bcr-abl mRNA have a privilege.

The efficiency of the designed ds/siRNAs was compared with that of Glivec. Glivec was applied in a dose of 180 nM every two days (as shown in Fig. 1C). To guarantee an equal effect of ds/siRNA-mix and Glivec on the cell proliferation activity of K-562 cells, the treatment with Glivec was carried out every two days and the effects on the parameters analyzed were detected on the 8th day. The results in Fig. 3 demonstrate that Glivec decreased markedly protein tyrosine kinase activity (~73%) and cell proliferation capacity (~54%), and provoked a comparatively poor decrease of bcr-abl protein (~14%).

Recently, we demonstrated that relatively long-term "chronic" application (6 days and more) of anti-bcr-abl oligo-DNAs markedly reduced the level of bcr-abl protein, but their sequence-specific effect was accompanied with influence of telomeric-associated proteins, increased telomerase activity and restoration of cell proliferation capacity in the treated cells [8,9]. Therefore, the comparatively long-term siRNA interference of bcr-abl oncogene has also a potential to influence positively the expression of other oncogenes, as well as factors, responsible for the proliferation and immortalization of bcr-abl-positive cells.

Similar effects have also been reported for Glivec. Brummendorf et al. [22] found an extension of the telomeres in CML-patients after long-term treatment with Glivec in both chronic phase and blast crisis. Analyzing 517 samples from 206 patients, the authors have been established that telomere length from start of treatment up to day 144 is significantly shorter compared with patients treated for more than 144 days. In patients with repeated measurements, a significant increase in telomere length under treatment is observed. Median telomere length in major remission has been found to be significantly longer in comparison with patients without

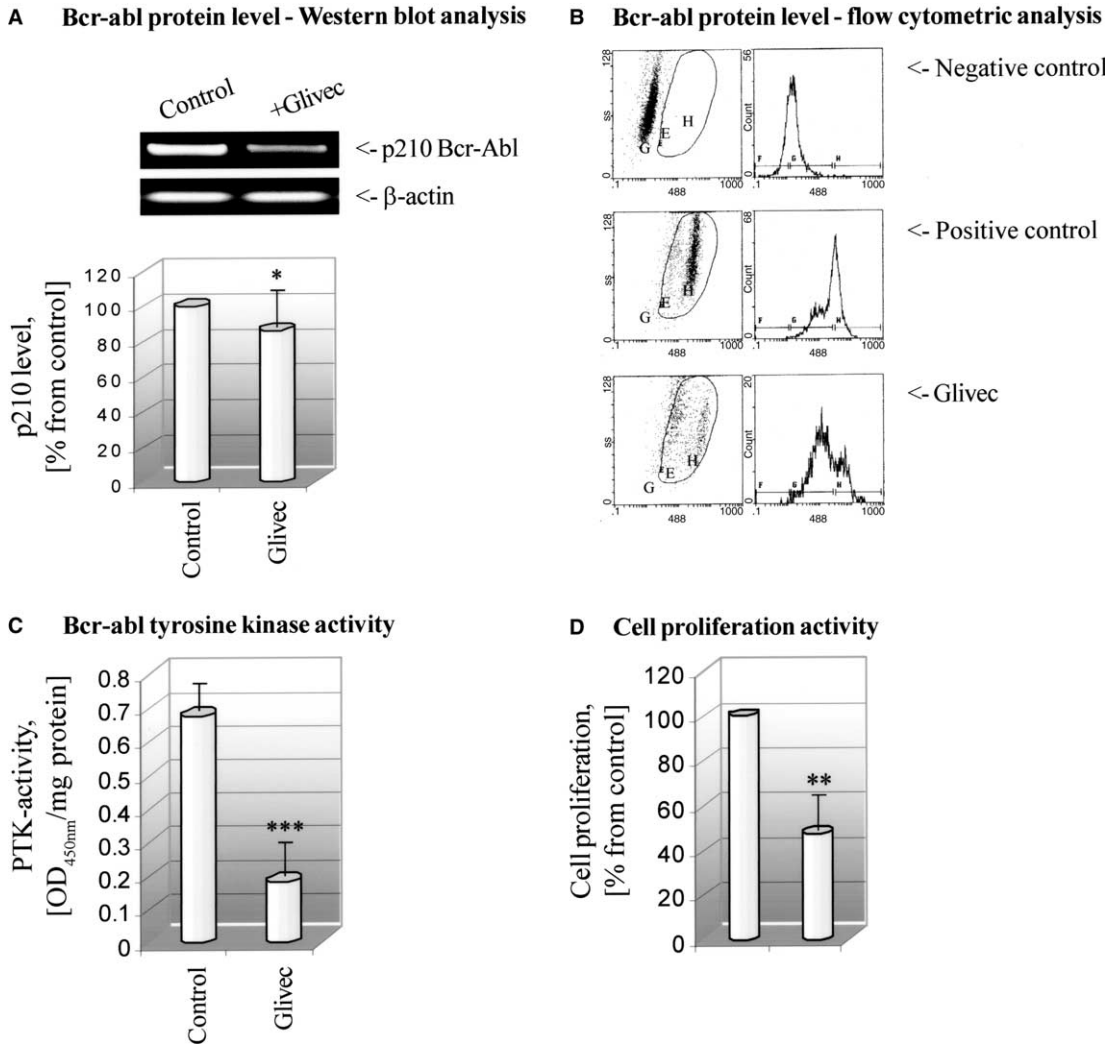


Fig. 3. (A) Effect of Glivec on the level of bcr-abl oncoprotein in K-562 cells – Western blot analysis. The cells were treated with Glivec and cell lysates were obtained after 8 days treatment as described in Section 2. The samples (in equal protein concentration) were dissolved 1:1 in 2× Laemmli buffer, heated at 95 °C for 10 min and subjected to SDS–polyacrylamide gel electrophoresis (80 V, 15 min; 120 V, 2 h at RT). The separated protein fractions were transferred to a Hybond-P PVDF membrane overnight at 35 V (4 °C). The membranes were cut at 45 kDa (for β-actin) and 210 kDa (for bcr-abl) and were incubated as follows: 1 h at RT in a blocking solution, 1 h in anti-c-abl antibody (rabbit, Sigma, 1:100 for detection of bcr-abl) or in anti-β-actin antibody (mouse, Calbiochem, 1:20 000), 1 h with HRP-conjugated goat anti-rabbit IgG (Sigma, 1:5000) for anti-c-abl or goat anti-mouse IgM (Sigma, 1:2000) for anti-β-actin. Chemiluminescence was detected immediately using ECL Advance Western Blotting Detection kit (Amersham Bioscience). Blots from one typical experiment for each protein are shown in the figure. Control blots are representative for untreated cells. In the histograms, the results are expressed as a percentage from the control group. The value of p210 bcr-abl protein in control was considered as 100%. The data are means ± S.D. from four independent experiments. * $P < 0.05$ vs. control group. (B) Effect of Glivec on the level of bcr-abl oncoprotein in K-562 cells – flow cytometric analysis. The cells were treated with Glivec as described in Section 2. After 8 days treatment, the cells were analyzed for the level of bcr-abl oncoprotein. Anti-c-abl antibody (rabbit, Sigma) was labeled by ZenonAlexa Fluor-488 Rabbit IgG Labeling kit (Molecular Probes). Permeabilization of cells for the Fluor-488-conjugated anti-c-abl was carried out by IntraPrep Permeabilization Reagent (Immunothech). The antibody–antigene interaction was detected by flow cytometry. Data are presented as a dot plot of Fluor-488 fluorescence (side scatter, SS – *y*-axis, vs. Fluor-488-conjugated antibody – *x*-axis in 1–1000 scale) with quadrant markers drawn to distinguish the cells, containing different levels of antibody–antigene complexes. In the histograms, quadrant H corresponds to the cells, containing maximum level of bcr-abl (positive control), quadrant G corresponds to the cells without fluorescent marker and therefore without bcr-abl–antibody complexes (spontaneous cell fluorescence, negative control), and the section between H and G (mentioned as E) corresponds to the cells, containing moderate or low level of Fluor-488-conjugated c-abl antibody and therefore expressing moderate or low levels of bcr-abl oncoprotein in comparison with the positive control. Histograms from one typical experiment are shown in the figure. Positive control is representative for Glivec-untreated cells. (C) Effect of Glivec on the protein tyrosine kinase activity in K-562 cells. The cells were treated with Glivec as described in Section 2 and cell lysates were obtained after 8 days treatment. Bcr-abl oncoprotein was isolated together with c-abl by immunoprecipitation, using anti-c-abl antibody (Sigma, 3 μg/ml) and protein A/G agarose beads. The tyrosine kinase activity of immunoprecipitated enzymes was analyzed, using CHEMICON's PTK Assay kit. The fraction of phosphorylated substrate was visualized spectrophotometrically at 450 nm, using HRP-conjugated anti-phosphotyrosine antibody (clone PY20) and ensuing chromagenic substrate reaction. The data are presented as means ± S.D. from six independent experiments. $OD_{450\ nm} = 1.0$ corresponds to 20 ng P-Tyr-peptide substrate. The calibration curve was linear in the range 10–40 ng P-Tyr-peptide substrate. *** $P < 0.001$ vs. control group. (D) Effect of Glivec on the cell proliferation activity of K-562 cells. The cells were cultured in the presence or in the absence of Glivec as it was described in Section 2. The cell proliferation activity was detected spectrophotometrically after 8 days treatment, based on the reduction of MTS into a formazan product (absorbance at 490 nm). The data were calculated as a percentage from control non-treated cells. The cell proliferating activity of control cells was considered as 100%. The results are presented as means ± S.D. from eight independent experiments. ** $P < 0.01$ vs. control group.

response to treatment, measured either by cytogenetics, interphase FISH, or quantitative RT-PCR.

Obviously, both pathways of bcr-abl suppression – siRNA-mediated cleavage of bcr-abl mRNA and Glivec-mediated inhibition of already synthesized fusion protein, are in a cross-talk with other oncogenes, apoptotic/antiapoptotic and cell proliferation factors. Both mechanisms keep a potential for restoration of the proliferation and immortalization of leukemia cells. To verify the factors potentially responsible for side-effects of Glivec and bcr-abl RNAi during long-term treatment, we provided a microarray analysis of gene profile in K-562 cells, ''chronically'' treated with ds/siRNA-mix or Glivec.

The expression of ~162 and ~104 genes was found to decrease and increase (>3 times), respectively, in ds/siRNA-treated cells versus 94 and 76 genes in Glivec-treated. The data in Tables 1 and 2 summarize the significant changes in

Table 1
Transcripts of interest suppressed >3 times in siRNA- or Glivec-treated K-562 cells – microarray analysis ($P < 0.01$)

Accession number	Description	siRNA-treated/ non-treated	Glivec-treated/ non-treated
Factors, responsible for cell proliferation and immortalization			
U47077	Human DNA-dependent protein kinase catalytic subunit	–	5.359
M23102	Human trk proto-oncogene insert of pLM6	4.941	–
U51869	Human proto-oncogene Bcd orf1 and orf2 mRNA	4.461	3.115
AF152961	Human chromatin-specific transcription elongation factor FACT 140 kDa subunit mRNA	4.371	3.903
AJ000052	Human gene encoding splicing factor SF1, exons 2–8	3.982	3.234
D29767	Human mRNA for Tec protein tyrosine kinase	3.859	3.594
X07109	Human mRNA for protein-kinase C-beta II	–	3.634
D51465	Nuclear factor I/X (CCAAT-binding transcription factor)	3.545	–
NM_00325	Tight junction protein 1	3.343	–
AF017789	Human putative transcription factor CA150 mRNA	–	3.336
T36282	Guanylate kinase	–	3.334
M54968	Human k-ras oncogene protein mRNA	–	3.301
L23320	Human replication factor C large subunit mRNA	3.310	–
S49592	E2F transcription factor	3.255	–
M34057	Latent transforming growth factor beta binding protein 1	3.232	–
AU118354	ATP-binding cassette, sub-family G (WHITE), member 2	3.188	–
M32721	Hyman poly(ADP-ribose)-polymerase mRNA	–	3.150
M64347	Human novel growth factor receptor mRNA	3.046	–
AB005659	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	3.015	–
Factors, responsible for apoptosis induction			
AF249273	Human Bcl-2-associated transcription factor	4.501	3.044
BI545796	Protein phosphatase 2, regulatory subunit B	–	4.453
Y13247	Protein phosphatase 1, regulatory subunit 10	–	4.111
U03106	Human wild-type p53 activated fragment-1	4.310	–
AB002331	Death-associated transcription factor 1	3.241	–
NM_03334	Caspase-7, apoptosis-related cysteine protease	3.109	–

Table 2
Transcripts of interest overexpressed >3 times in siRNA- or Glivec-treated K-562 cells – microarray analysis ($p < 0.01$)

Accession number	Description	siRNA-treated/ non-treated	Glivec-treated/ non-treated
Factors, responsible for cell proliferation and immortalization			
M24779	Human protein kinase-related oncogene (PIM1) mRNA	–	9.913
AA232339	Pim-1 oncogene	4.889	–
AA863383	Pim-2 oncogene	–	3.074
BM020481	Tumor protein D52-like 1	4.673	–
J04111	Human c-jun proto oncogene, clone hCJ-1	4.323	4.490
X52125	Human alternatively spliced c-myb mRNA, clone pMbm-1	–	4.496
X52479/AF035594	Protein-kinase C-alpha mRNA for PK-C-alpha	4.171	3.134
AI498125	Pvt-1 oncogene homolog, MYC activator	4.104	3.881
U80017	Human basic transcription factor 2 p44 (MAPK activator)	–	3.565
BG387620	v-myb myeloblastosis viral oncogene homolog	3.452	–
M96956	Teratocarcinoma-derived growth factor, clone CR-3	3.404	–
AF053949	Human transcription factor CBFA1/OSF2 gene	3.046	–
X52125	Human alternatively spliced c-myb mRNA	–	4.496
Factors, responsible for apoptosis induction			
M54894	Interleukin-6 (interferon, beta 2)	12.38	5.915
X57351	Human 1-8D gene from interferon-inducible gene family	5.098	4.707
AA769631	Tumor necrosis factor receptor superfamily, 10b	4.954	4.429
AF090693	Human apoptosis-related RNA binding protein	4.546	–
J04164	Human interferon-inducible protein 9-27	–	3.590
BC010399	STAT induced STAT inhibitor-2	3.402	3.780
NM00218	Interleukin-13	–	3.341

genes of interest – kinases, apoptotic/antiapoptotic factors, cell proliferating factors and oncogenes. Table 1 includes the transcripts with decreased expression in ds/siRNA- or Glivec-treated K-562 cells (>3 times). RNAi of bcr-abl was accompanied with decreased expression of several proto-oncogenes (trk and Bcd orf1/orf2 proto-oncogenes), growth factors (novel growth factor receptor, latent transforming growth factor β binding protein 1), factors relating to kinase activity (Tec protein tyrosine kinase, ATP-binding cassette – sub-families C-CFTR/MRP and G-WHITE) and factors relating directly to cell proliferation cycle (Table 1). Several factors responsible for development of apoptosis also increased significantly (>3 times): STAT induced STAT inhibitor-2, apoptosis-related RNA binding protein (NAPOR-3), tumor necrosis factor receptor superfamily (member 10b), interleukin 6 (Table 2).

However, 6 days treatment of K-562 cells with anti-bcr-abl ds/siRNA-mix resulted in a significant decrease of several apoptotic factors (caspase-7, p53, death associated transcription factor 1) and antiapoptotic factors (bcl-2-associated transcription factor) (Table 1), as well as in a significant increased expression of the following transcripts that can be responsible for restoration of the cell proliferation (Table 2): protein kinase-related oncogene (PIM1), c-jun proto-oncogene, Pvt-1 oncogene homologue (myc-activator), protein kinase C-α gene, teratocarcinoma-derived growth factor 3, transcription factor CBFA1/OSF2.

Eight days treatment of K-562 cells with Glivec was accompanied with suppression of Bcd orf1/orf2 proto-oncogene, k-ras oncogene, factors relating to kinase activity (Tec protein tyrosine kinase, protein kinase C-β) and factors relating directly to cell proliferation cycle (Table 1). Glivec also suppressed protein phosphatase 1 and 2 genes and thus can stimulate indirectly the activity of other protein kinases. Significant overexpression of the following transcripts was detected in Glivec-treated K-562 cells (Table 2): protein kinase-related oncogene (PIM2), c-jun proto-oncogene, Pvt-1 oncogene homologue (myc-activator), protein kinase C-α gene, human basic transcription factor 2 p44 (MAPK activator). However, Glivec induced an overexpression of several apoptotic factors as: interleukins 6 and 13, 1-8D gene from interferon-inducible gene family, tumor necrosis factor receptor superfamily (10b), interferon-inducible protein 9-27, STAT-induced STAT-inhibitor 2.

In summary, the results from microarray analysis demonstrate that siRNA interference of bcr-abl expression and inhibition of already synthesized bcr-abl fusion protein by Glivec are in a cross-talk with several common factors that might be responsible in major degree for cell death or for restoration of the cell proliferation after long-term treatment.

Both, anti-bcr-abl RNAi and Glivec-treatment of K-562 cells decreased in equal degree bcl-2-associated transcription factor (bcl-2 is one of the major apoptotic signals), and overexpressed in equal degree c-jun proto-oncogene, protein kinase C-α, pvt-1 oncogene homologue (myc-activator) (all they are well-known antiapoptotic factors). It can hypothesized that bcr-abl oncoprotein participates in the regulation of these common genes and/or their respective proteins. To guarantee the success of siRNA interference and Glivec, it is necessary to clarify the expression of all these factors and to prognosticate their role in restoration of cell proliferation during long-term treatment. It did not exclude many other genes to be influenced significantly during treatment more than 6–8 days as in the present study. In this context, the combined application of Glivec (or anti-bcr-abl siRNAs) with other substances directed to the genes described above is a promising strategy for CML control.

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