Small interfering RNA against *BCR-ABL* transcripts sensitize mutated T315I cells to nilotinib

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ABSTRACT

Background

Selective inhibition of the BCR-ABL tyrosine kinase by RNA interference has been demonstrated in leukemic cells. We, therefore, evaluated specific *BCR-ABL* small interfering RNA silencing in BCR-ABL-positive cell lines, including those resistant to imatinib and particularly those with the T315I mutation.

Design and Methods

The factor-independent 32Dp210 *BCR-ABL* oligoclonal cell lines and human imatinib-resistant *BCR-ABL*-positive cells from patients with leukemic disorders were investigated. The effects of *BCR-ABL* small interfering RNA or the combination of *BCR-ABL* small interfering RNA with imatinib and nilotinib were compared with those of the ABL inhibitors imatinib and nilotinib.

Results

Co-administration of *BCR-ABL* small interfering RNA with imatinib or nilotinib dramatically reduced BCR-ABL expression in wild-type and mutated *BCR-ABL* cells and increased the lethal capacity. *BCR-ABL* small interfering RNA significantly induced apoptosis and inhibited proliferation in wild-type ($P<0.0001$) and mutated cells (H396P, T315I, $P<0.0001$) *versus* controls. Co-treatment with *BCR-ABL* small interfering RNA and imatinib or nilotinib resulted in increased inhibition of proliferation and induction of apoptosis in T315I cells as compared to imatinib or nilotinib alone ($P<0.0001$). Furthermore, the combination of *BCR-ABL* small interfering RNA with imatinib or nilotinib significantly ($P<0.01$) reversed multidrug resistance-1 gene-dependent resistance of mutated cells. In T315I cells *BCR-ABL* small interfering RNA with nilotinib had powerful effects on cell cycle distribution.

Conclusions

Our data suggest that silencing by *BCR-ABL* small interfering RNA combined with imatinib or nilotinib may be associated with an additive antileukemic activity against tyrosine kinase inhibitor-sensitive and resistant BCR-ABL cells, and might be an alternative approach to overcome BCR-ABL mutations.

Key words: small interfering RNA, CML, BCR-ABL.

Introduction

Chronic myeloid leukemia (CML) is a relatively well differentiated myeloproliferative disorder originating from transformed hematopoietic stem cells. 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*).[1,2] Treatment with imatinib mesylate, a selective inhibitor of ABL, the chimeric BCR-ABL fusion protein, platelet-derived growth factor receptor (PDGF-R) α and β, and stem cell factor receptor (c-KIT), has shown remarkable clinical activity with minimal side effects in *BCR-ABL*+, *C-KIT*+ or *PDGFR*+ malignancies and resulted in complete cytogenetic remission in a majority of CML patients in chronic phase, but in fewer patients with accelerated and blast phase disease.[3,4] However, despite the impressive efficacy of imatinib, resistance to this drug can emerge and is a significant clinical issue. In the phase III International Randomized Interferon *versus* STI571 (IRIS) trial, an estimated 5% of patients with newly diagnosed chronic phase-CML failed to achieve a complete hematologic response at 3 months, 16% failed to achieve a major cytogenetic response with imatinib at 12 months, and 24% failed to achieve a complete cytogenetic response at 18 months. The estimated relapse rate of patients in this trial was 17% and 7% of patients developed disease progression.[5] Although allografting is still considered to be a curative option in CML, it is associated with significant mortality and morbidity, thus the number of transplants performed for this disease has dropped dramatically since imatinib became available.[6] To overcome resistance, strategies such as dose escalation, combination with conventional drugs (cytarabine, interferon), alternative BCR-ABL inhibitors, and BCR-ABL protein down-regulating agents have been adopted.[7]

Nilotinib (ANM-107, Tasigna®, Novartis Pharmaceuticals Corp.) is a high-affinity aminopyrimidine-based ATP-competitive inhibitor that decreases proliferation and viability of wild-type BCR-ABL and imatinib-resistant BCR-ABL mutant-expressing cells *in vitro* by selectively inhibiting BCR-ABL autophosphorylation. Nilotinib is more potent than imatinib as an inhibitor of BCR-ABL in a wide range of CML-derived and transfected cell lines.[8-10] In 2007 the U.S. Food and Drug Administration granted accelerated approval for the use of nilotinib in the treatment of chronic and accelerated phase Philadelphia chromosome-positive CML in adult patients resistant or intolerant to prior therapy that included imatinib.[11] Gene targeting of *BCR-ABL* fusion transcripts is an ideal way to selectively kill those cells, leaving normal cells unaffected.

RNA interference is an evolutionarily conserved cellular mechanism that mediates sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA. Small interfering RNA (siRNA) are the mediators of mRNA degradation in the process of RNA interference. Synthetic siRNA are able to mediate cleavage of the target RNA, as shown by Elbashir *et al.*[12] Scherr *et al.* published a study showing that siRNA directed against *BCR-ABL* can specifically inhibit BCR-ABL expression in Philadelphia chromosome-positive cell lines and cells from CML patients.[13]

Furthermore, Wohlbold *et al.* showed that siRNA treatment might sensitize cells to imatinib contributing to its therapeutic potential.[14] We demonstrated that combined transfection with Wilms' tumor 1 gene (*WT1*) siRNA and *BCR-ABL* siRNA in Philadelphia chromosome-positive cell lines and cells of CML patients enhanced inhibition of cell proliferation and induction of apoptosis compared to transfection with *BCR-ABL* siRNA or *WT1* siRNA alone.[15] In addition, we showed that *BCR-ABL* siRNA had anti-proliferative and pro-apoptotic effects on Philadelphia chromosome-positive AML cells *in vitro*.[16] Importantly, we demonstrated the feasibility of targeted, non-viral delivery of *BCR-ABL* siRNA as a therapeutic approach in a female CML patient with imatinib-resistant bone marrow and extramedullary relapse after allogeneic hematopoietic stem cell transplantation.[17]

In this study, we investigated the activity of the two protein tyrosine kinase inhibitors imatinib and nilotinib. We compared these agents to *BCR-ABL* siRNA in several murine bcr-abl-positive cell lines which differ in their sensitivity to imatinib or nilotinib and in human imatinib-resistant *BCR-ABL*-positive cells from patients with acute leukemias.

Design and Methods

Cell culture

The factor-independent 32Dp210 *BCR-ABL* oligoclonal cell line was generated by transfection of parental cells with the retroviral vector Migp210, Migp210-Thr315Ile, or Migp210-His396Pro, as previously described.[18,19] All transfected 32Dp210 cell lines were a generous gift from Dr. H. van der Kuip (Stuttgart, Germany) and Prof. Dr. J. Duyster (Munich, Germany). The cells were grown in RPMI 1640 medium (Invitrogen, Heidelberg, Germany) supplemented with 10% fetal bovine serum (FBS) complemented with glutamine as described. All cells were maintained in a humidified 37°C incubator with 5% CO_2.

Leukemia cells from bone marrow of patients were also used. All patients gave informed written consent to the donation and use of their cells.

Drug dosing and administration

Imatinib (IM; Gliveec®) and nilotinib (Tasigna®) were kindly provided by Novartis Pharma AG (Novartis, Germany). A stock solution of imatinib (10 mg/mL) was prepared by dissolving the compound in dimethylsulfoxide (DMSO): H_2O (1:1) and was then stored at -20°C. Imatinib was used at a concentration of 1 µM for 32Dp210-wt and 32Dp210-His396Pro cells and at 1 or 3 µM for 32Dp210Thr315Ile cells. A stock solution of nilotinib (10 mg/mL) was prepared by dissolving the compound in DMSO:H_2O (1:1) and this too was stored at -20°C. Nilotinib was used at a concentration of 1 µM for 32Dp210wt and 32Dp210-His396Pro cells and 1 or 3 µM for 32Dp210-Thr315Ile cells. For the tests using a combination of *BCR-ABL* siRNA with imatinib or nilotinib, cells were treated with imatinib or nilotinib and 54 pM siRNA for 24-48 h.

Transfection of small interfering RNA

Sequences of siRNA directed against the *BCR-ABL* transcript were published by Scherr *et al.*[13] The siRNA was purchased from Qiagen-Xeragon (Hilden, Germany) with the sense 5`-GCAGAG UUCAAAAGCCCUUdTdT and antisense 5`-AAGGGCUUU UGAACUCUGCdTdT and a scrambled siRNA sequence was used

as a control (sense 5`-UUGUACGGCAUC AGCGUUAdTdT and antisense 5`-UUACGUGAUGCCGUA CAAdTdT). *In vitro* transfections with 0.8 μg (54 pM) siRNA were performed in 24-well plates using the DOTAP® liposomal transfection reagent (1×10^5 cells/well) (Roche Applied Science, Indianapolis, USA) following the manufacturer's protocol. Transfection of siRNA was performed at least five times in each experiment. Each experiment was repeated at least twice. As a control we used two or more non-silencing siRNA (mismatched or scrambled siRNA) from Qiagen.

RNA isolation and real-time reverse transcriptase polymerase chain reaction

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real time reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described for *BCR-ABL* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).[20] For *BCR-ABL* and 6-glucosephosphate dehydrogenase we used a commercial Light-Cycler®-t(9;22) Quantification Kit (Roche Molecular Diagnostics, Mannheim, Germany) following the manufacturer's protocol. For *MDR1* RT-PCR we used the following primers and hybridization probes: primers 5'-ggA AgC CAA TgC CTA TgA CTT TA-3´ and 5´gAA CCA Ctg CTT CgC TTT CTg-3´, hybridization probes 5'-6FAM-TgA AAC TgC CTC ATA AAT TTg ACA CCC Tgg-TAMRA. For quantification, RNA transcript expression was normalized by determining the ratio between expression levels of targets and *GAPDH*.

Growth inhibition assay

Cells were cultured in 96-well plates at a density of 5000 cells/well and left to recover. The quantity of viable cells was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (10 μL of 5 mg/mL solution, Sigma Chemical Co., Germany) was added to each well of the titration plate and incubated for 4 h at 37°C. The cells were then treated with DMSO (40 μL/well) and incubated for 60 min at 37°C. The absorbance of each well was determined in an enzyme linked immunosorbent assay (ELISA) 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 to untreated control cells.

Terminal transferase dUTP 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 instructions. 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 field were randomly counted for each sample. At least three samples with 15 single analyses in total were counted.

Cell proliferation assay

Cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) incorporation. Twenty-four hours (and for up to 48 h) after transfection of siRNA, the 1×10^5 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. Proliferation was determined from the percentage of brown stained cells among the total number of cells in each sample with the analyses done in the same way as for apoptotic cells.

Cell cycle analysis

Cells were fixed in ice-cold ethanol (70% v/v) and stained with a solution of propidium iodide (PI) (20 μg/mL PI; 100 μg/mL RNase; 0.1% Triton X-100; and in 870 μg/mL trisodium citrate; Sigma Chemical, Germany) The DNA content was determined using a FACScan flow cytometer (Coulter, Krefeld, Germany). Cell-cycle distribution was analyzed using MultiCycle for Windows (Phoenix Flow System, San Diego, USA). Red fluorescence (585 ± 42 nm) was evaluated on a linear scale, and pulse width analysis was used to exclude cell doublets and aggregates from the analysis. Cells with a DNA content between 2N and 4N were designated as being in the G1, S, or G2/M phase of the cell cycle. The number of cells in each compartment of the cell cycle was expressed as a percentage of the total number of cells present. In sequential combination experiments, cells were pretreated with *BCR-ABL* siRNA or mismatched *BCR-ABL* siRNA for 24-48 h, washed twice with phosphate-buffered saline to remove the siRNA, or without *BCR-ABL* siRNA and analyzed for cell cycle distribution.

Flow cytometry

Between 1.0×10^5 to 5.0×10^5 cells were labeled with antibody for multi-color flow cytometry using phycoerythrin- or peridin chlorophyll-conjugated monoclonal antibodies directed against CD45, anti-phosphotyrosine protein (p-Tyr, PY99) and 7-amino-actinomycin D (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 added for 30 min at room temperature in the dark. After incubation with the primary antibody, cells were washed twice with buffer and incubated with phycoerythrin cynanin 5-labeled goat anti-rabbit IgG secondary antibody. Cells were again washed and resuspended in 500 μL of phosphate-buffered saline for flow cytometry analysis. All antibodies were obtained from Beckmann-Coulter (Krefeld, Germany) or Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Non-specific 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, Krefeld, Germany).

Statistics

Differences in data from the various groups were tested by the two-tailed unpaired *t* test or Mann-Whitney U-test using the SPSS 14 program (SPSS Inc, Chicago, USA).

Results

Efficiency of transfection in 32Dp210 BCR-ABL *cell lines and in primary leukemic cells of different* BCR-ABL-*positive acute leukemic patients*

Using fluorescence-marked, non-silencing siRNA (Qiagen) we evaluated the transfection rate in 32Dp210 *BCR-ABL* oligoclonal cells and in primary *BCR-ABL* positive leukemic cells. Twenty-four hours after transfection the number of fluorescence-marked cells was quantified using a fluorescence microscope. For this, 100 cells were counted in quintuplicate. We found a mean ± standard deviation (SD) transfection rate of 74.4±7.6% in 32Dp210 *BCR-ABL* cells and a mean transfection rate of 51.8±11.8% in primary *BCR-ABL* positive leukemic cells.

Inhibition of cell growth in 32Dp210 BCR-ABL cell lines and leukemic cells of BCR-ABL- positive acute leukemia patients

To assess whether treatment with imatinib, nilotinib, *BCR-ABL* siRNA or the combination of these drugs with *BCR-ABL* siRNA affects the viability of *BCR-ABL*-expressing leukemic cells, these were transfected with *BCR-ABL* siRNA, scrambled siRNA or without siRNA with constant doses (54 pM) of siRNA or incubated with imatinib or nilotinib for 24-48 h, harvested, and then cell viability analyzed by an MTT assay. As shown in Figure 1A, imatinib, nilotinib, *BCR-ABL* siRNA and the combination of imatinib or nilotinib with *BCR-ABL* siRNA significantly decreased the cell viability in 32Dp210-wt cells. Imatinib, nilotinib and the combination with siRNA had the strongest effects on reducing the viability of 32Dp210 *BCR-ABL* cells to 24.6 - 27.4% (mean) of control levels. siRNA reduced the viability of these cells to 48.6%. The combination of siRNA with imatinib or nilotinib reduced cell viability to a similar extent as the treatment with a single tyrosine kinase inhibitor (imatinib or nilotinib) in 32Dp210-wt cells. The mismatched control *BCR-ABL* siRNA, even when used at a dose up to 108 pM, did not inhibit cell viability since 32Dp cells are not sensitive to the scrambled siRNA (*data not shown*).

Next, we studied whether the inhibition of *BCR-ABL* translation by siRNA is sufficient to overcome resistance to imatinib or nilotinib, as illustrated in Figure 1B and 1C. In 32Dp210-His396Pro cells, which render the respective cells 5-fold less sensitive to imatinib, we observed a marked reduction of cell viability with nilotinib and the combination

of nilotinib with *BCR-ABL* siRNA, to 46.3% and 31.3% of control values, respectively. siRNA treatment alone resulted in a moderate reduction of viability of the transfected cells to about 65%. The combination of imatinib and *BCR-ABL* siRNA could reduce cell viability to 42.7% that of control cells. The combination of imatinib with siRNA was more effective than *BCR-ABL* siRNA or nilotinib alone. A much greater inhibition of 32Dp210-His396Pro cells was found with the combination of nilotinib and *BCR-ABL* siRNA than with the combination of imatinib and *BCR-ABL* siRNA (*P*<0.02), as shown in Figure 1B. In the 32Dp cells with the Thr315Ile mutation, which are completely resistant to imatinib and nilotinib, only *BCR-ABL* siRNA and the two combinations of siRNA with either imatinib or nilotinib caused a significant reduction of cell viability to 50.3% and 63.5%, respectively. siRNA alone led to a moderate reduction in the viability of the transfected cells to about 64%. The combination of *BCR-ABL* siRNA with imatinib or nilotinib resulted in a powerful reduction compared with that achieved by siRNA alone (*P*<0.05) or resistant 32DP control cells (*P*<0.02). Nilotinib with *BCR-ABL* siRNA had the strongest effects, reducing the viability of 32Dp210-Thr315Ile cells to 50.3%, but this was not significantly different from the reduction achieved with the combination of imatinib and siRNA (*P*<0.062), as shown in Figure 1C.

In order to assess the biological significance of these findings, we subsequently studied the effects of *BCR-ABL* siRNA on cell growth. Cells from patients with imatinib-resistant leukemias were treated with 54 pM *BCR-ABL* siRNA. To exclude the possibility that effects were not specific to *BCR-*
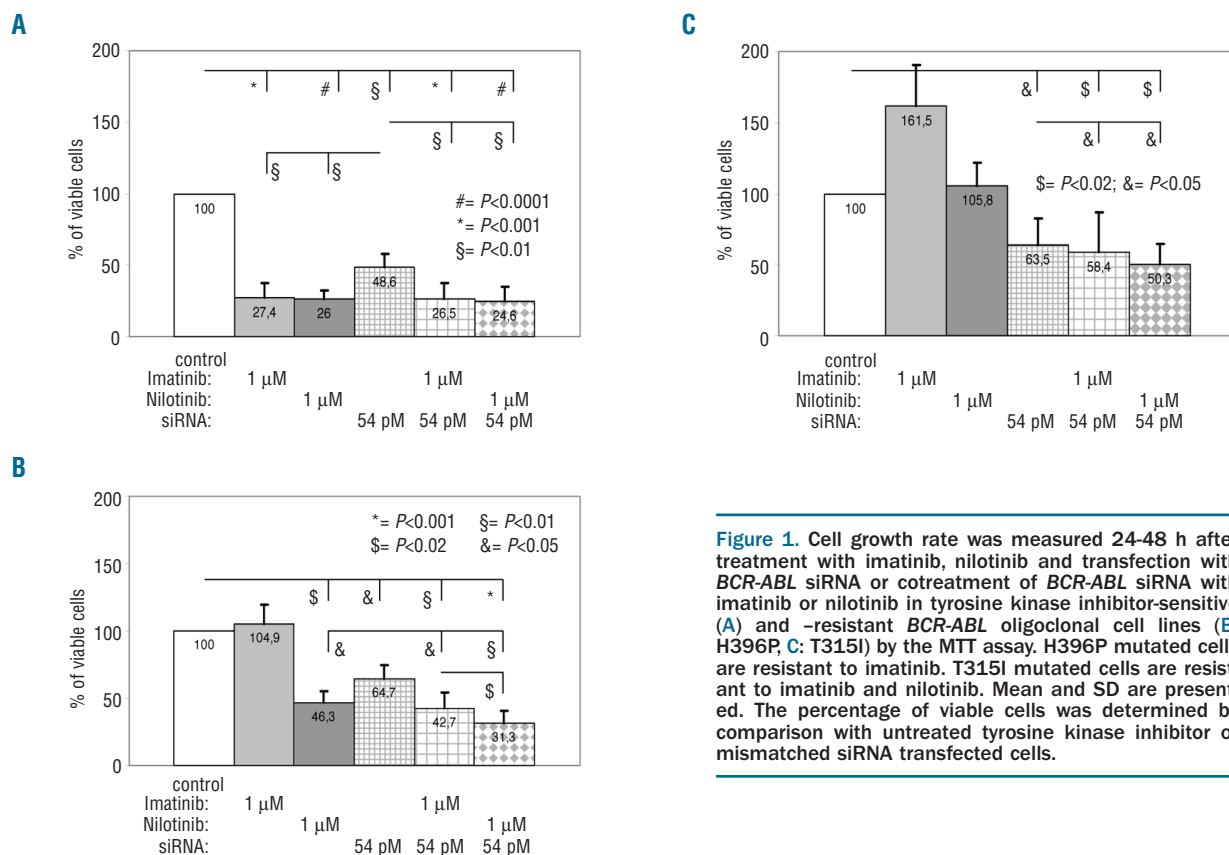
Figure 1. Cell growth rate was measured 24-48 h after treatment with imatinib, nilotinib and transfection with *BCR-ABL* siRNA or cotreatment of *BCR-ABL* siRNA with imatinib or nilotinib in tyrosine kinase inhibitor-sensitive (**A**) and –resistant *BCR-ABL* oligoclonal cell lines (**B**: H396P, **C**: T315I) by the MTT assay. H396P mutated cells are resistant to imatinib. T315I mutated cells are resistant to imatinib and nilotinib. Mean and SD are presented. The percentage of viable cells was determined by comparison with untreated tyrosine kinase inhibitor or mismatched siRNA transfected cells.

ABL siRNA, we performed a control experiment using a scrambled *BCR-ABL* siRNA up to a maximal dose of 108 pM for 48 h, followed by harvest and analysis of cell viability, as presented in the *Online Supplementary Table S1*.

Taken together, these data show that *BCR-ABL* siRNA inhibits the growth of both imatinib-resistant 32Dp cells and cells from patients with imatinib-resistant hematologic malignancies.

BCR-ABL *gene expression measured by real time reverse-transcriptase polymerase chain reaction analysis*

We found a marked reduction of *BCR-ABL* expression in 32Dp210-wt cells, which was quantified by correlation to the housekeeping gene *GAPDH* by real-time RT-PCR (ratio of *BCR-ABL/GAPDH*). The levels of *BCR-ABL* mRNA were reduced to between 54.7% and 31.9% (mean) after treatment with imatinib, nilotinib or *BCR-ABL* siRNA transfection in comparison with controls (set at 100%), as shown in *Online Supplementary Figure S1A*. The combination of a tyrosine kinase inhibitor with *BCR-ABL* siRNA produced a further reduction of *BCR-ABL* mRNA to 36.2% when using the siRNA with imatinib and to 15.8% when using siRNA combined with nilotinib. The combination of *BCR-ABL* siRNA with nilotinib was, indeed, significantly more effective than siRNA combined with imatinib ($P<0.01$).

In 32Dp210-His396Pro cells we found a significant reduction of *BCR-ABL* mRNA levels to 40.3% of control levels after nilotinib treatment and to 46.4% after transfection with the *BCR-ABL* siRNA. The combination of either imatinib or nilotinib with *BCR-ABL* siRNA reduced the *BCR-ABL* mRNA to levels ranging from 29.1% to 31.2% in 32Dp210-His396Pro cells (*Online Supplementary Figure S1B*). Again, the combination of nilotinib with *BCR-ABL* siRNA caused a significantly greater reduction, to 29%, compared with that achieved by imatinib combined with siRNA ($P<0.05$).

In 32Dp210-Thr315Ile cells, a significant reduction of *BCR-ABL* mRNA to 60.4% was seen after transfection with siRNA. Combining the *BCR-ABL* siRNA with imatinib resulted in a reduction to 46.6% compared with a reduction to 40.9% when siRNA was co-administered with nilotinib ($P<0.05$ for imatinib with siRNA compared to nilotinib with siRNA) (*Online Supplementary Figure S1C*). To assess the biological significance of these findings, we subsequently studied the effects of *BCR-ABL* siRNA on *BCR-ABL* mRNA levels of the several patients, as presented in the *Online Supplementary Table S1*. Taken together, these data show that *BCR-ABL* siRNA inhibits the expression of *BCR-ABL* mRNA in both imatinib-resistant 32Dp cells and in cells from patients with imatinib-resistant hematologic malignancies.

Phosphotyrosine protein and phospho-Crkl expression measured by flow cytometry

To verify the above described findings, we next performed flow cytometric studies on the effects on phosphotyrosine protein (p-Tyr) and phosphor-Ckrl (p-Crkl) expression, accepted as an excellent method for assessing BCR-ABL status, in the tyrosine kinase inhibitor resistant 32Dp210Thr315Ile cells. We found a significant reduction of p-Tyr expression to 69% or 66% after high doses (up to 3 μM) of imatinib or nilotinib ($P<0.01$). We did not, however, detect any reduction in p-Crkl expression in 32Dp210-Thr315Ile cells after treatment with high doses of imatinib or

nilotinib (*Online Supplementary Figure S2*). The administration of *BCR-ABL* siRNA reduced the level of p-Tyr to 48.2% of that in untreated control cells ($P<0.0001$) and the level of p-Crkl to 45.9% ($P<0.001$). The combination of nilotinib with *BCR-ABL* siRNA also achieved significant reduction of the tyrosyl-phosphoproteins, to 35.9% for p-Tyr ($P<0.05$) and to 5.4% for p-Crkl ($P<0.001$), as compared to *BCR-ABL* siRNA alone. Comparing the combination of imatinib and *BCR-ABL* siRNA (reduction to 11.8%) with that of nilotinib and *BCR-ABL* siRNA (reduction to 5.4%), the latter had greater efficacy in reducing p-Crkl expression in 32Dp210-Thr315Ile cells ($P<0.02$). Taken together, these data suggest that the combination of nilotinib and *BCR-ABL* siRNA results in enhanced inhibition of tyrosyl-phosphoproteins.

Cell proliferation measured by 5-bromo-2-deoxyuridine

We determined whether treatment with imatinib, nilotinib, *BCR-ABL* siRNA or the combination of siRNA with either imatinib or nilotinib affects cell proliferation. Cell proliferation was determined by a 5-bromo-2-deoxyuridine (BrdU) incorporation assay following the manufacturer's instructions. The control was set at 100%. In 32Dp210-wt cells, the administration of imatinib, nilotinib or siRNA alone significantly decreased cell proliferation. The greatest reduction, to 11.8% (mean), occurred with imatinib, whereas the administration of nilotinib and *BCR-ABL* siRNA caused reductions to 15.2% and 15.8%, respectively ($P<0.0001$). The combination of siRNA with either imatinib or nilotinib further reduced proliferation to 7.2% and 10.6% ($P<0.0001$), respectively. In 32Dp210-His396Pro cells, the administration of imatinib led to a lesser reduction in proliferation, as expected, to only 60.0% ($P<0.001$), whereas nilotinib and siRNA led to reduction in proliferation to 14.2% and 17.4%, respectively ($P<0.0001$). Despite the limited effect of imatinib alone, the combination of imatinib with siRNA led to the most impressive reduction to 6.2% ($P<0.0001$), whereas the combination of siRNA with nilotinib caused a reduction to 7.2% ($P<0.0001$). In 32Dp210-Thr315Ile cells only *BCR-ABL* siRNA ($P<0.0001$) and combinations of siRNA with imatinib and nilotinib caused significant reductions of cell proliferation to 19.6% and 10.2%, respectively ($P<0.0001$). The combination of nilotinib with *BCR-ABL* siRNA reduced the proliferation rate of the resistant 32Dp210-Thr315Ile cells to 10.2% ($P<0.01$) of the proliferation rate of these cells after treatment with imatinib combined with siRNA (Table 1).

Effect on cell apoptosis, determined by terminal transferase dUTP nick-end labeling assay

We further investigated the effect of imatinib, nilotinib, or *BCR-ABL* siRNA alone or in combination with either imatinib or nilotinib on cell apoptosis (Table 2). Numbers of apoptotic cells were determined using an *in situ* cell death kit according to the manufacturer's instructions. The increase in apoptosis was compared to a control level set at one. Apoptosis of 32Dp210-wt cells was increased from 4.5 to 9.8-fold by the various treatment strategies: imatinib and siRNA, when administered alone, induced 4.9-fold and 4.5-fold increases in apoptosis, whereas their combination increased apoptosis by 7.1-fold ($P<0.0001$) as compared to control levels. Effects were greater with nilotinib which, alone, induced a 7.3-fold increase and in combina-

tion with the siRNA resulted in a 9.8-fold increase in apoptosis ($P<0.0001$).

We also examined the apoptotic effect of the drugs administered to 32Dp210-His396Pro cells, which are 5-fold less sensitive to imatinib, and to the fully resistant 32Dp210-Thr315Ile cells. With regards to 32Dp210-His396Pro cells, the administration of imatinib alone failed to induce significant apoptosis, whereas either nilotinib or siRNA alone or the combination of siRNA with either imatinib or nilotinib induced significant, 5.0 to 7.0-fold increases in apoptosis ($P<0.0001$). Similarly, in 32Dp210-Thr315Ile cells only the administration of siRNA alone or in combination with imatinib or nilotinib induced significant, 4.9-to 5.3-fold increases in apoptosis ($P<0.0001$ for all), whereas the administration of imatinib or nilotinib alone failed to induce significant apoptosis.

The combination of nilotinib with *BCR-ABL* siRNA induced the greatest increase in apoptosis (7.0-fold, $P<0.01$) compared to all other treatments in the less sensitive 32Dp210-His396Pro cells. In the 32Dp210-Thr315Ile cells, the increase in apoptosis was limited to approximately 5-fold (Table 2).

Effects of imatinib, nilotinib, short interfering RNA or their combinations on cell cycle

We next investigated the influence of imatinib, nilotinib, *BCR-ABL* siRNA or the combination of siRNA with either imatinib or nilotinib on the cell cycle, i.e., the relative distribution of cells in the G1, S, G2 and sub-G1 phases. In 32Dp210-wt cells, the highest percentage of cells in the apoptotic sub-G1 phase was found following treatment with nilotinib alone (36.8%) or in combination with siRNA (41.9%). The sub-G1 values with imatinib alone, siRNA alone and the combination of imatinib and siRNA were significantly lower (range, 6.9-14.4%). Interestingly, nilotinib and the combination of siRNA with nilotinib led to a reduction of cells in S-phase (decreases to 66.7% and 72.9%) and a moderate reduction of cells in G0/G1-phase to 10% and 18%, respectively. The percentage of cells in G2/M-phase ranged from 2.5% to 5%. With imatinib alone, siRNA alone and the combination of siRNA with imatinib, the percentages of cells in the G0/G1 and G2/M phases changed moderately, whereas cells in S-phase decreased to 20.8% after treatment with imatinib or the combination of siRNA with imatinib.

In 32Dp210-His396Pro cells, which are 5-fold less sensitive to imatinib, the effect of imatinib alone or the combination of imatinib and siRNA on the apoptotic sub-G1 values were, as expected, less. Furthermore, the effect of siRNA on 32Dp210-His396Pro cells was lower than that on wild-type cells. Again, nilotinib alone and in combination with siRNA was more effective, inducing the highest numbers of cells in the sub-G1 phase (17.8% and 28.3%, respectively), and reducing those in S-phase (to 52.1% and 52.7%, respectively). We observed moderate changes in the distribution of cells in the G0/G1 and G2/M phases after treatment with nilotinib alone or in combination with siRNA. Imatinib alone, siRNA alone and the combination of siRNA with imatinib had moderate effects on cell cycle distribution.

Much smaller effects were seen in the completely imatinib-resistant 32D cell line with the Thr315Ile mutation: cells were induced into the apoptotic sub-G1 phase after transfection with siRNA or the combination of siRNA with imatinib or nilotinib (5.6% for siRNA, 8.0% for siRNA with imatinib, and 12.7% for siRNA with nilotinib, compared to the controls, imatinib or nilotinib). Interestingly, the greatest reduction of the S-phase was achieved with *BCR-ABL* siRNA alone and in combination with nilotinib (42.5% and 42.1%, respectively, compared to controls). The distribution of cells in all other phases of the cell cycle was changed moderately in 32Dp210-Thr315Ile cells (*Online Supplementary Figure S3*).

Effects on multiple drug resistance, assessed by real-time polymerase chain reaction

It has been reported that the *MDR1* gene and its product, Pgp protein, are major obstacles to more successful therapy for CML. We, therefore, analyzed *MDR1* gene expression relative to expression levels of the housekeeping gene *GAPDH* (Figure 2). The control was set at 100%. In 32Dp210-wt cells, imatinib decreased *MDR1* levels slightly (86%, mean), whereas nilotinib and siRNA had greater effects (reductions to 42.5% and 46.5%, respectively). The lowest *MDR1* levels were observed with the combinations of siRNA and imatinib (32.2%), and siRNA with nilotinib (9.9%).

In 32Dp210-His396Pro cells, we found significant effects of nilotinib (78.6%) and siRNA (82.5%), although treatment with the combination of siRNA with either imatinib or nilotinib caused significantly greater reductions in *MRD1* levels (to 20.7% and 17.4% of control levels, respectively). This effect was also pronounced in 32Dp210-Thr315Ile cells, with co-administration of siRNA with either imatinib or nilotinib producing the lowest *MRD1* levels (72.7% to 69.6%). Treatment with *BCR-ABL* siRNA alone resulted in a mean reduction of *MRD1* level to 86.6%, whereas after treatment with either imatinib or nilotinib the *MRD1* levels increased to 151.3% and 136.6%, respectively. Interestingly, the most significant reduction of *MRD1* levels in 32Dp210-Thr315Ile cells was achieved with nilotinib combined with *BCR-ABL* siRNA (69.6%), which was significantly greater than that achieved by *BCR-ABL* siRNA alone ($P<0.01$). Taken together, these data show that combining *BCR-ABL* siRNA with nilotinib can strongly influence the expression of *MDR1* in both imatinib-sensitive and imatinib-resistant 32Dp210 cells.

Table 1. Proliferation rate, measured by BrdU incorporation after treatment with imatinib, nilotinib and transfection with *BCR-ABL* siRNA or co-treatment of *BCR-ABL* siRNA with imatinib or nilotinib in tyrosine kinase inhibitor-sensitive and –resistant *BCR-ABL* oligoclonal cell lines.

Treatment	Culture cells		
	32Dp210-wt	32Dp210-His396Pro	32Dp210-Thr315Ile
Imatinib	11.8±3	60.0±9	126.3±9
Nilotinib	15.2±3	14.2±3	104.6±7
BCR-ABL siRNA	15.8±4	17.4±3	19.6±4
BCR-ABL siRNA with imatinib	7.2±3	6.2±2#	17.8±6
BCR-ABL siRNA with nilotinib	10.6±4	7.2±2#	10.2±2

Control was set at 100%. Values are mean ± SD: See text for statistical significances.

Discussion

Given that siRNA can now be delivered effectively to mammalian cells, selective interventions in leukemic cell gene regulation might be feasible for the treatment of leukemia.[13,21] There are increasing numbers of reports of *in vitro* and *in vivo* selective silencing of the *BCR-ABL* fusion gene by RNA interference.[15-17,22] Importantly, this construct was found to induce target-specific *BCR-ABL* mRNA cleavage without affecting the expression of *BCR* or *ABL* mRNA.[14] Moreover, the first study on *BCR-ABL* siRNA showed that *BCR-ABL* silencing was accompanied by strong induction of apoptotic cell death.[22] The rate of induced apoptosis was as high as that induced by 1 μM imatinib. Other investigators have confirmed these effects of *BCR-ABL* siRNA on CML cells.[14,23,24]

In the current study, we found that siRNA directed against the *BCR-ABL* gene significantly inhibited *BCR-ABL* gene expression in factor-independent 32Dp210 *BCR-ABL* oligoclonal cell lines, including those resistant to imatinib (particularly those with the T315I mutation). The response to increasing doses of *BCR-ABL* siRNA reached a plateau at 60% in the resistant 32Dp210-Thr315Ile cells, and the maximum *BCR-ABL* gene reduction in the 32Dp210 cells was two-fold as compared to the controls. As expected, we found differential reductions of *BCR-ABL* gene expression in response to two tyrosine kinase inhibitors (imatinib and nilotinib) in 32Dp210 cell lines. The results of *BCR-ABL* gene expression reduction and inhibition of cell proliferative capacity were concordant. O'Hare *et al.* compared the *in vitro* activity of BCR-ABL inhibitors in clinically relevant imatinib-resistant *ABL* domain mutants, and showed higher efficacy of nilotinib in many imatinib-resistant cell lines, with the exception of the T315I mutant.[25] In a phase II study, nilotinib produced hematologic and cytogenetic responses in imatinib-resistant and -intolerant patients with CML in accelerated phase with the exception of those expressing the T315I mutation.[26] In this study, we recorded large reductions in the growth of 32Dp210 cells (72.6% for imatinib, 74% for nilotinib, and 51.4% for siRNA) and smaller, but still significant reductions of as much as 35% for *BCR-ABL* siRNA in imatinib-resistant 32Dp210-His396Pro cells and imatinib-resistant 32Dp210-Thr315Ile cells. Consistent with the findings of Wohlbold *et al.*,[27] who showed that BCR-ABL can be completely down-regulated by RNA interference in all common p210 and p190 BCR-ABL variants, we found reduced cell growth rates and *BCR-ABL* expression in primary leukemic cells from *BCR-ABL*-positive patients with acute lymphoblastic and myeloid leukemia and CML after transfection with *BCR-ABL* siRNA. Furthermore, we detected relevant differences in siRNA delivery to primary cells and to 32Dp cell lines. These results might, to some extent, reflect differences in the effectiveness of transfection with *BCR-ABL* siRNA in primary cells and 32Dp cell lines, which we found to vary from 52% and 75%. Furthermore, the expression of BCR-ABL protein is 5- to 6-fold higher in 32Dp210 cell lines than in primary cells.[14] It should be emphasized that the efficacy of siRNA-mediated gene silencing is affected by a combination of factors (variation in transfection efficiency, siRNA sequences, properties of the target mRNA, and others). Moreover, Hu *et al.*[28] suggested that low-abundant transcripts are less susceptible to siRNA-mediated degradation than medium- and high-abundant transcripts.

Surprisingly, we found that co-administration of *BCR-ABL* siRNA with imatinib or nilotinib resulted in enhanced inhibition of cell growth and *BCR-ABL* gene expression in 32Dp210 cells, including cells with the T315I mutation, which are highly resistant to imatinib and nilotinib. This might be the result of effective modulation by a breakpoint-specific siRNA. Consistent with our findings it was previously reported that transfection with *BCR-ABL*-specific siRNA increased the sensitivity of both imatinib-sensitive and imatinib-resistant CML cell lines to imatinib.[14] Furthermore, Baker *et al.* showed that the IC$_{50}$ of imatinib was 3-fold lower in K562 cells transfected with synthetic or recombinant-generated *BCR-ABL* siRNA enabling imatinib to efficiently inhibit the high levels of BCR-ABL protein found in these cells.[29]

Complex signal transduction pathways are also involved in controlling the proteolysis of potential BCR-ABL effectors that may be responsible for some features of BCR-ABL-transformed cells such as deregulated cellular proliferation and apoptosis control through effects on multiple intracellular signaling pathways, including the Ras, phosphatidylinositol 3-kinase (PI3K), JAK-STAT, and NF-κB pathways.[30] For example, Perrotti *et al.* found that the levels of TLS/FUS, an RNA/DNA-binding protein involved in transcriptional and post-transcriptional regulation of gene expression, are markedly increased in growth factor-dependent hematopoietic cell lines ectopically expressing BCR-ABL and in CML blast crisis bone marrow cells.[31] In addition to regulating gene expression at the transcriptional level, it is increasingly clear that BCR-ABL is also involved in post-transcriptional regulation via shuttling of heterogeneous nuclear ribonucleoproteins (hnRNP). These proteins play an important role in the control of pre-mRNA processing, nuclear mRNA export, and translation.[32]

Notably, the inhibitory effect on intracellular p-Tyr, measured by flow cytometry, declined significantly in the resistant 32Dp210-Thr315Ile cells after treatment with high doses of imatinib or nilotinib or transfection with *BCR-ABL* siRNA or the co-administration of siRNA with imatinib or nilotinib in comparison with that of mismatched siRNA or non-manipulated controls. Moreover, the inhibitory effect on p-Crkl was significantly correlated with siRNA treatment and with co-treatment with *BCR-ABL* siRNA and imatinib or

Table 2. Apoptosis rate, measured by terminal transferase dUTP nick-end labeling assay, after treatment with imatinib, nilotinib and transfection with *BCR-ABL* siRNA or co-treatment with *BCR-ABL* siRNA and imatinib or nilotinib in tyrosine kinase inhibitor-sensitive and –resistant *BCR-ABL* oligoclonal cell lines.

| Treatment	Culture cells		
	32Dp210-wt	32Dp210-His396Pro	32Dp210-Thr315Ile
Imatinib	4.9±0.5	1.5±0.1	1.1±0.2
Nilotinib	7.3±0.5	5.5±0.2	1.0±0.1
BCR-ABL siRNA	4.5±1.9	5.3±0.6	4.9±0.5
BCR-ABL siRNA with imatinib	7.1±1.2	5.0±0.7	5.0±0.8
BCR-ABL siRNA with nilotinib	9.8±1.1	7.0±0.2	5.3±0.2

Control was set at 1.0. Values are mean ± SD. See text for statistical significances.

nilotinib ($P<0.0001$). The tyrosine kinase activity of BCR-ABL constitutively induces the phosphorylation of a large panel of proteins, activating multiple signaling pathways responsible for protection against apoptosis, stimulation of growth factor-independent proliferation, and alterations of cellular adhesion. P-Crkl, a BCR-ABL adaptor protein, serves as a surrogate for BCR-ABL kinase activity *in vivo*.[33] Our data on p-Crkl and p-Tyr support the efficacy of *BCR-ABL* siRNA transfection *in vitro* and validate our above described findings on cell growth and *BCR-ABL* expression in mutated 32Dp210 cells. Furthermore, we found that combining *BCR-ABL* siRNA transfection with imatinib or nilotinib had additive effects on induction of apoptosis and inhibition of proliferation of 32Dp210 cell lines, including mutated 32Dp210-His396Pro cells and mutated 32Dp210-Thr315Ile cells. The combination reduced the proliferation rate by about 2-2.5 fold compared to the use of *BCR-ABL* siRNA alone ($P<0.01$). This suggests that *BCR-ABL* siRNA and tyrosine kinase inhibitors affect *BCR-ABL*-positive cells by modulating or transforming genes via different pathways promising additive selective antitumor activity.

Ong *et al.* recently reported that mammalian target of rapamycin (mTOR)-dependent pathways are constitutively activated in BCR-ABL-transformed cells.[34,35] These studies raised the possibility that regulation of mTOR may be a mechanism by which BCR-ABL promotes cell growth and survival of leukemic cells, but the mTOR effectors mediating such responses are not fully understood. Tyrosine kinase inhibitors, such as imatinib and nilotinib, target and block engagement of the mTOR pathway in BCR-ABL-expressing

cells. Such effects of BCR-ABL kinase inhibitors are observed in cells transformed by imatinib-resistant BCR-ABL kinase mutants, establishing that the mTOR/p70 S6K pathway plays a critical role in promoting the leukemogenic effects of BCR-ABL.[36] Recently, Ohba *et al.* demonstrated, by microarray analysis of K562 cells, that there is cross-talk between siRNA interference and *BCR-ABL* oncogeny and found that the expression of approximately 250 genes was changed. RNA interference of *BCR-ABL* was accompanied by decreased expression of various proto-oncogenes, growth factors, factors related to kinase activity, and factors related directly to cell proliferation. Several factors responsible for the development of apoptosis increased, including STAT-induced STAT inhibitors and apoptosis-related RNA binding proteins.[37] Furthermore, Zhou *et al.* demonstrated that Adelson helper integration site 1 (Ahi-1/AHI-1) is a novel BCR-ABL interacting protein that is also physically associated with JAK2. This AHI-1-BCR-ABL-JAK2 complex seems to modulate BCR-ABL-transforming activity and the response/resistance of CML stem/progenitor cells to tyrosine kinase inhibitors through an interleukin-3-dependent BCR-ABL and JAK2-STAT5 pathway.[38]

In experiments focused on cell cycle analysis, the cell cycle distribution and apoptotic responses induced by *BCR-ABL* siRNA, tyrosine kinase inhibitors or co-treatment with *BCR-ABL* siRNA and tyrosine kinase inhibitors seemed to differ. We found that the effects of transfection with *BCR-ABL* siRNA depend on cell cycle arrest in the early S phase rather than on the disruption of the G2 checkpoint and on an increased apoptotic distribution with DNA fragmentation
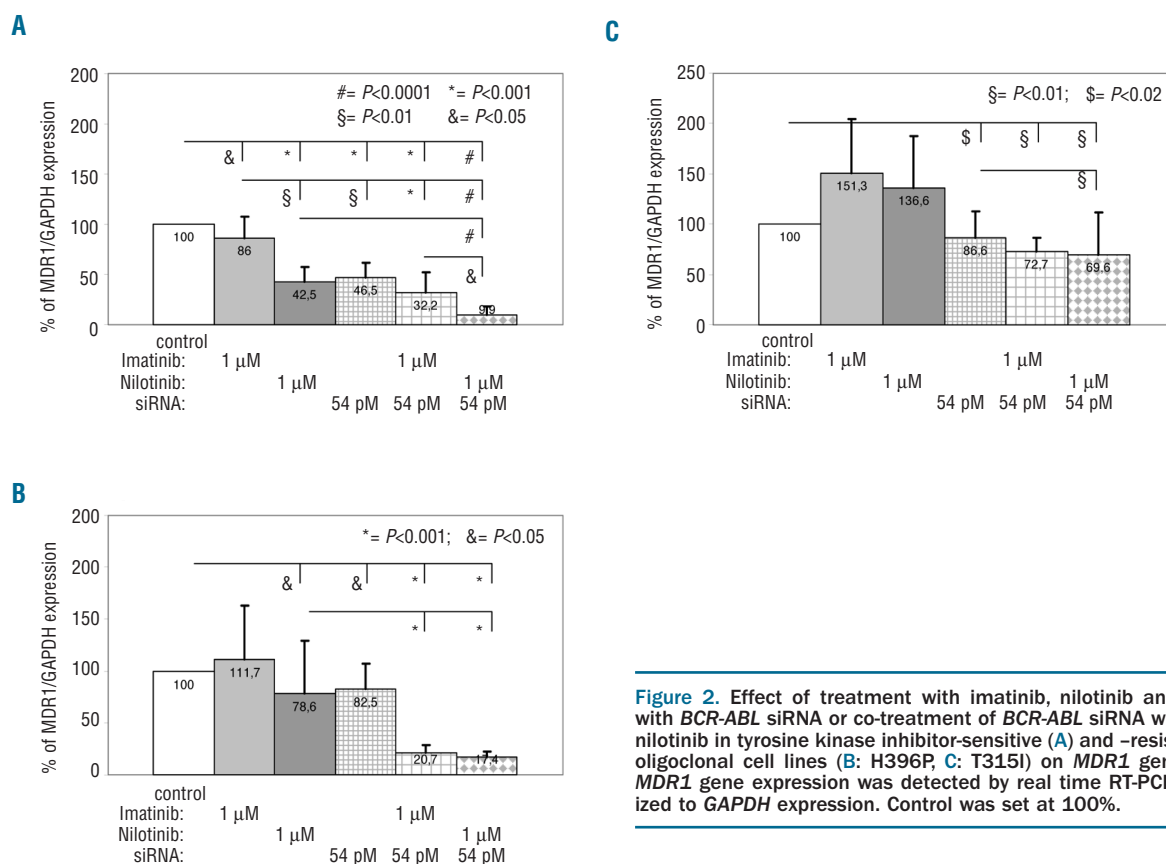
Figure 2. Effect of treatment with imatinib, nilotinib and transfection with *BCR-ABL* siRNA or co-treatment of *BCR-ABL* siRNA with imatinib or nilotinib in tyrosine kinase inhibitor-sensitive (A) and –resistant *BCR-ABL* oligoclonal cell lines (B: H396P, C: T315I) on *MDR1* gene expression. *MDR1* gene expression was detected by real time RT-PCR and normalized to *GAPDH* expression. Control was set at 100%.

(sub-G0/G1 phase). By adding imatinib or nilotinib to *BCR-ABL* siRNA treatment these effects increased approximately 1.3-fold (*P*<0.05). This strongly suggests that dual inhibition of *BCR-ABL* by *BCR-ABL* siRNA combined with nilotinib additively enhances 32Dp210 cytotoxicity and apoptosis. In K562 cells, imatinib enhanced the inhibitory effect of radiation on proliferation of K562 cells and decreased the G2 phase content due to an efficient arrest in the S phase.[39] Mazzacurati *et al.* found that imatinib also induces growth arrest by activating the Chk2-Cdc25A-Cdk2 axis, a pathway complementary to p53 in the activation of the G1/S cell cycle checkpoint, involved in cell fate decisions such as regulation of cell cycle progression, DNA repair and survival rather than apoptosis.[40] Other investigators suggested that alternate pathways in addition to apoptosis may lead to the inhibition of cell growth mediated by tyrosine kinase inhibitors.[41,42]

There are several pharmacological mechanisms by which CML cells develop resistance to tyrosine kinase inhibitors, such as reduced drug-uptake and increased drug-efflux-related surface molecules including MDR-1, which reportedly mediates the efflux of imatinib. Such mechanisms may also lead to the resistance of other tyrosine kinase inhibitors, such as nilotinib.[43-45] Specifically, a predominant acquisition of MDR1-dependent resistance is found in the majority of patients with advanced CML and other leukemias, as well as in patients during relapse.[46,47] These pharmacokinetic mechanisms of resistance to tyrosine kinase inhibitors may occur in addition or may induce the well-described pharmacodynamic mechanisms involving the *BCR-ABL* gene in patients with CML.[48] It should be stressed that our real-time RT-PCR data show a highly significant reduction of more than 13% in *MDR1* gene expression (*P*<0.02) after transfection with *BCR-ABL* siRNA in mutated 32Dp210Thr315Ile cells. The combination of *BCR-ABL* siRNA with imatinib or nilotinib resulted in an additional reduction of *MDR1* gene expression of 27% or 30% (*P*<0.01). In addition to impaired drug transport, aberrant activation of signal transduction proteins, including nuclear factor-κB, has been implicated in treatment failure in hematologic malignancies including CML.[49,50] Our study demonstrates that *BCR-ABL* siRNA reliably and effectively induces apoptosis and inhibition of proliferation in factor-independent 32Dp210 *BCR-ABL* oligoclonal cell lines, including those resistant to imatinib and particularly those with the T315I mutation. Moreover, additive effects of *BCR-ABL* siRNA with tyrosine kinase inhibitors, such as imatinib or nilotinib, result in anti-proliferative and enhanced pro-apoptotic effects in 32Dp210 cells. In addition, the combination of *BCR-ABL* siRNA with imatinib or nilotinib significantly reversed MDR1-dependent multidrug resistance of mutated 32Dp210 cells. Modulation of breakpoint-specific siRNA by agents such as tyrosine kinase inhibitors might open the door to new important alternative or complementary approaches for the treatment of BCR-ABL-expressing hematologic disorders.

Authorship and Disclosures

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