Efficient Silencing of *bcr*/*abl* Oncogene by Single- and Double-Stranded siRNAs Targeted against b2a2 Transcripts†

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ABSTRACT: In this work, double- and single-stranded small-interference RNAs (siRNAs) were designed to knock down the *bcr*/*abl* oncogene in leukaemia KYO-1 cells. The siRNA molecules were targeted against two distinct sites encompassing the b2a2 junction of the *bcr*/*abl* transcripts. The siRNAs were able to reduce the levels of both *bcr*/*abl* mRNA and protein $p210^{BCR/ABL}$. Conversely, control siRNAs bearing 3 or 4 base-pair substitutions did not produce any inhibitory effect. The designed siRNAs were also found to be active in KCl22 cells, which harbor the b2a2 junction, but not in K562 cells, which, by contrast, harbor the b3a2 junction. The anti-b2a2 siRNAs promoted biological effects on KYO-1 cells, because the *bcr*/*abl* suppression resulted in the inhibition of cell growth and colony formation in agar and activation of apoptosis and upregulation of the cell-cycle inhibitor p27 protein. The bioactivity of the designed siRNAs is discussed in terms of internal stability of the RNA duplexes. Our data suggest that siRNAs can be considered strong tools for functional analysis of *bcr*/*abl* and for developing molecular therapeutic approaches to leukaemia.

A reciprocal translocation between chromosomes 9 and 22, t(9;22), is found in more than 95% of patients with chronic myeloid leukaemia (CML)[1] and in about 20% of patients with acute lymphoblastic leukaemia (ALL) (*1*−*3*). This translocation fuses the 5′ portion of the *bcr* gene in chromosome 22 with the 3′ portion of the *abl* gene in chromosome 9, generating an aberrant chromosome 22, named Philadelphia chromosome, which contains the chimeric *bcr*/*abl* gene (*4*, *5*). Breakpoints in the *abl* gene occur 5′ to exon 2. *abl* exons 2−11 (called a2−a11) are transposed into the major breakpoint cluster region of *bcr*, located between exons 12 and 16 (called b1 and b5) (*6*). The breakpoint in *bcr* occurs 5′ between b2 and b3 or 3′ between b3 and b4. A chimeric *bcr*/*abl* gene with either a b2a2 or a b3a2 junction is formed (*6*). It encodes for a protein of 210 kDa ($p210^{BCR/ABL}$), which is localized in the cytoplasm and involved in the leukemogenesis (*2*). Protein $p210^{BCR/ABL}$ is a tyrosine kinase, like ABL, but while the latter is normally regulated, the former shows an uncontrolled activity that usurps the physiological functions of the ABL protein. Protein $p210^{BCR/ABL}$ assumes a dimeric conformation and interacts with a variety of molecules resulting in a deregulated cellular proliferation, a reduced apoptotic response to mutagenic stimuli, and a decreased adherence of leukaemic cells to bone marrow stroma (*7*). Because the cellular effects of $p210^{BCR/ABL}$ are exerted through its interaction with a number of proteins pertaining to different cellular pathways, *bcr*/*abl* represents a fundamental target to set up molecular therapies. Because b2a2 and b3a2 junctions are present only in CML cells, the corresponding transcripts are attractive targets.

Previous attempts to suppress the expression of *bcr*/*abl* with antisense (*8*−*10*), ribozyme (*11*), PNA (*12*), and antigene oligonucleotide (*13*) have not been completely satisfactory, neither for therapeutic purposes, nor for functional genomic studies. Recently, it has emerged that specific genes can be knocked down by double-stranded RNAs. This is a natural phenomenon called RNA interference (RNAi), occurring in plants and animals, that probably has the function to defend organisms against viruses (*14*−*17*). Ever since Tuschl and co-workers (*14*) demonstrated that synthetic small-interference RNA (siRNA) duplexes, long 21−23 nucleotides, are able to trigger the RNAi pathway, the use of synthetic RNA duplexes for post-transcriptional gene silencing is rapidly growing. Indeed, they enable gene function studies without the need to produce knockout mice, which are expensive and difficult to obtain. Recently, we have used siRNA to inhibit the expression of *bcr*/*abl* in leukaemic cells. Although a couple of studies propose useful guidelines for the selection of highly effective siRNAs, the identification and validation of efficient RNAi targets remains a fundamental step to obtain an efficient suppression of the target gene. Some recent reports describe the use of siRNAs to suppress oncogene *bcr*/*abl* in leukaemic cells (*18*−*20*). The siRNAs used were specific for b3a2 transcript, and up to now, b2a2 transcripts have not yet been considered as siRNA targets. However, it is noteworthy that CML cells have normally either the b2a2 or b3a2 transcripts, and in 5% of the cases, alternative splicing events allow the expression of both types of fusion transcripts (*21*). Therefore, there is a great interest to search for molecules capable to

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[1] Abbreviations: CML, chronic myeloid leukaemia; ALL, acute lymphoid leukaemia; siRNA, small interference RNA; PCR, polymerase chain reaction; RT, reverse transcriptase.

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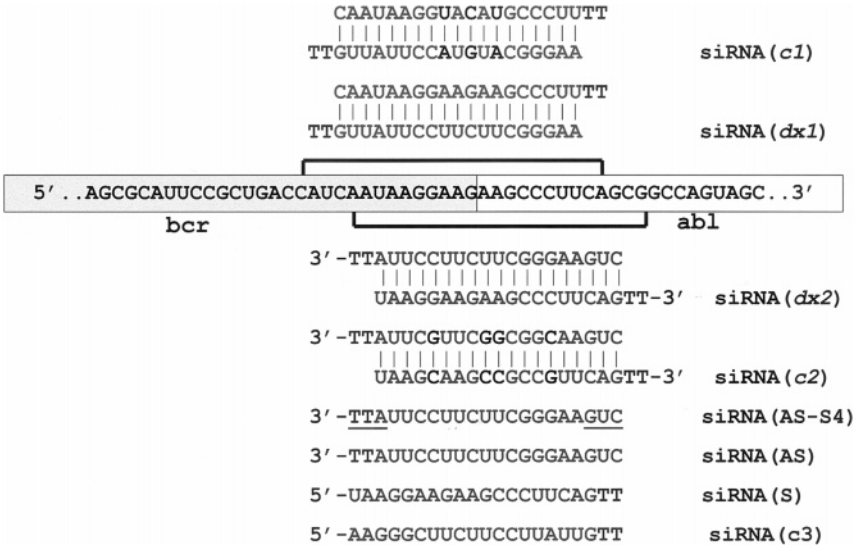

FIGURE 1: Sequences of the *bcr*/*abl* b2a2 junction and designed anti-b2a2 [siRNA(*dx1*), siRNA(*dx2*), siRNA(*AS-S4*), and siRNA(*AS*)] and control [siRNA(*c1*), siRNA(*c2*), siRNA(*c3*), and siRNA(*S*)] siRNAs. The siRNA targets on *bcr*/*abl* mRNA are shifted by three nucleotides, one with respect to the other. TT indicates 3′-overhang deoxythymidine dimers. 5′ and 3′ ends of siRNA(*AS-S4*) each contain two phosphorothioate linkages (underlined bases).

specifically suppress b2a2 transcripts of CML cells. In our work, we tested in different cells the bioactivity of both double- and single-stranded siRNAs designed to recognize specific targets within the b2a2 junction. Our results show that both double- and single-stranded siRNAs efficiently silence the *bcr*/*abl* gene, because the levels of b2a2 mRNA and protein $p210^{BCR/ABL}$ are strongly suppressed in the treated cells. Moreover, we show that the suppression of *bcr*/*abl* induces apoptosis and impairs cell growth in KYO-1 cells. Our data suggest that siRNAs offer a new approach to molecular cancer therapy and also provide a formidable tool to knockout gene expression for functional genomic studies.

MATERIALS AND METHODS

Cell Culture. CML cells K562 (a gift of Dr. M. Giunta, Genetic Unit, University of Udine, Italy), KYO-1, and KCl22 (a gift of Dr. C. M. Broughton, Department of Hematology, University of Liverpool, U.K.) were maintained in exponential growth in RPMI 1640 medium containing 100 units/mL penicillin, 100 mg/mL streptomycin, 200 mM L-glutamine, and 10% fetal bovine serum (FBS) (Celbio, Milan, Italy), which was heat-inactivated at 56 °C for 20 min.

siRNA. The siRNAs used in this study, specific for the b2a2 junction region of *bcr*/*abl* were purchased from PROLIGO Primers and Probes (Paris, France). Their sequences are illustrated in Figure 1. The annealing of sense and antisense strands to form the RNA duplexes (20 μM) was performed in the hybridization buffer (25 mM Tris-HCl at pH 7.5 and 100 mM NaCl) by heating the samples to 68 °C for 10 min and slowly cooling to room temperature. Cell transfection was performed with Oligofectamine Reagent (Invitrogen, Life Technologies, Milano, Italy), according to the optimized procedure recommended by M. Wilda (*18*) and by electroporation (25 μF, 500 V, ∞ Ω) using an Bio-Rad gene pulser. In every experiment, the dose of different siRNA used is 100 nM.

Flow Cytometry. To study the cellular uptake KYO-1 cells were transfected with FITC-labeled single- and double-stranded siRNAs, in the same way as the other experiments with or without oligofectamine and with electroporation. Aliquots of the cell populations after transfection were spun down by centrifugation at 1000 rpm and 4 °C, washed and resuspended in phosphate-buffered saline (PBS), and analyzed by flow cytometry (Becton Dickinson).

Confocal Microscopy. After transfection with fluorescein-conjugated siRNA, the cells were harvested at 5 and 24 h, centrifuged, washed twice with PBS, spun on a glass slide, and fixed with 3% paraformaldehyde (PFA) in PBS for 20 min. After the cells were washed with 0.1 M glycine, containing 0.02% sodium azide in PBS, to remove PFA, and Triton X-100 (0.1% in PBS), they were incubated with 24 μg/mL propidium iodide and 0.4 mg/mL RNase A for 30 min at 37 °C to stain the nuclei. Then, coverslips were mounted on the glass slide with mowiol 4-88 and DABCO (2.5%). The cells were analyzed using a Leica DM IRBE confocal imaging system. Diaphragm and fluorescence detection levels were adjusted to minimize any interference between the fluorescein and propidium iodide channels.

Cell Proliferation Assay. The cells were counted daily, and the viability was assessed by trypan blue exclusion, following a standard procedure.

RNA Transcript Isolation and cDNA Synthesis. mRNA was isolated using GenoPrep Direct mRNA Kit by GenoVision (NO-0884, Oslo, Norway). cDNA synthesis was performed as follows: a volume of 5 μL of mRNA solution in DEPC-treated water was heated at 70 °C and cooled on ice. The solution was added to 20.6 μL of cDNA mix [10 μL of 5$\times$ buffer and 5 μL of 0.1 M dithiothreitol (Life Technologies, Milan, Italy), 2 μL of a 12.5 pmol primer AZ (5′-CCATTTTTGGTTTGGGCTTCACACCATTCC; MWG Biotech), 0.6 μL of 40 units/mL RNAse inhibitor (Life Technologies, Milan, Italy), and 1 μL of 200 units of M-MLV reverse transcriptase (Life Technologies)]. The reactions (50 μL/tube) were incubated for 1 h at 37 °C. As a negative control, the reverse transcription reaction was performed with 5 μL of DEPC-treated water. The cDNA was stored at −20 °C.

Polymerase Chain Reaction (PCR). A volume of 5 μL of cDNA, heated at 95 °C for 5 min, was mixed with 10 μL of *bcr*/*abl* mix [5 μL of 10× Taq polymerase buffer (Euro-Clone), 0.75 μL of 25 mM $MgCl_2$, 1 μL of 12.5 pmol/μL primer EA122, 5′-GTTTCAGAAGCTTCTCCCTG, 1 μL of 12.5 pmol/μL primer EA500, 5′-TGTGATTATAGCCTAA-GACCCGGAG (MWG-Biotech), 1 μL of 5 mM dNTPs with equimolar amounts of dTTP, dCTP, dATP, and dGTP (Euro-Clone), and 0.25 μL of 5 units/μL Taq DNA polymerase (Euro-Clone)]. Amplification was carried out on an automated DNA thermal cycler (Progene) as follows: 35 cycles of denaturation (94 °C for 30 s), annealing (60 °C for 30 s), and extension (72 °C for 30 s). *abl* amplification (annealing at 55 °C) was carried out with primers EA500 and ABL1A, 5′-CCTCTCGCTGGACCCAGTGA. The effect of siRNA on the level of b2a2 *bcr*/*abl* mRNA in siRNA-treated KYO-1 cells was quantified by competitive reverse transcriptase (RT)-PCR. Messanger RNA extracted from an equal number of K562 (competitor) and anti-b2a2 siRNA-treated KYO-1 (target) cells was subjected to RT-PCR, using primers EA122 and EA500. Two amplified *bcr*/*abl* bands of 387 and 312 bp were obtained from K562 and KYO-1 cells, respectively (*22*). As a control, we amplified a 128-bp DNA fragment of *abl*, using primers ABL1A and EA500.

Western Blotting. Western blot analyses were performed on total protein lysates in a 2× Laemmli sample buffer [3.3% sodium dodecyl sulfate (SDS), 22% glycerol, 1.1 M Tris-HCl at pH 6, 0.001% bromophenol blue, and 10% β-mercaptoethanol]. Samples were heated at 95 °C for 10 min and loaded in 8.5% resolving, 3.5% stacking SDS−polyacrylamide gel. Electrophoresis was carried out at 100 V for 20 min and 150 V for 2 h. The relative concentration of the protein lysates in each sample was estimated by a Markwell test (*23*) as well as by electrophoresis. Equal amounts of lysates were transferred to a nitrocellulose membrane (Sartorius, Germany) using a Dry Blot Transfer (EBU 202, C.B.S. Scientific Co.). The nitrocellulose membrane was colored with Ponceau and cut at the 27, 45, 210 kDa molecular weight levels. Each membrane was used to detect p27, β-actin, and *bcr*/*abl*, by means of a double antibody procedure. The membranes were incubated under agitation for 1 h at room temperature in blocking solution (PBS containing 5% dry milk and 0.1% Tween 20) and then at room temperature for 2 h in c-ABL (Ab-3, 1:40, Calbiochem) and β-actin monoclonal antibodies (Ab-1, 1:5000 Calbiochem) p27 (Ab-2, 1:80, Calbiochem). The antibody solution was removed, and the membranes were washed 3 times with PBS containing 0.1% Tween 20. The membranes were then incubated (1 h at room temperature under agitation) with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10 000) (Calbiochem) for the Ab-3 and Ab-2 and goat anti-mouse IgM (1:10 000) (Calbiochem) for the β-actin antibody. Chemiluminescence was detected immediately as described by the manufacturer (Super Signal West Pico and Dura Trial; Pierce). Films were exposed for about 10 min for the BCR/ABL protein, 45 min for p27, and 1 min for β-actin. Band intensities were measured with a LKB Ultrascan XL Enhanced Laser Densitometer (Bromma).

Apoptotic Assay. We performed Caspase activity assays using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega), according to the protocol of the manufacturer. Forty-eight hours following transfection, siRNA-treated KYO-1 cells, in triplicate, were seeded at a concentration of 5×10^4 cells (25 μL/well) into a 96-well plate. Then, Homogeneous Caspase-3/7 Reagent (the substrate was diluted 1:100 with the provided buffer) was added maintaining a 1:1 ratio of the reagent to the sample. The measure of fluorescence of each well was read at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm (Spectra Max Gemini XS, Molecular Devices Corporation, CA).

Colony Assay. Two days after transfection with the various siRNAs, for each sample, 10^4 cells were plated in 1 mL of semisolid medium (2% agar, RPMI containing 20% FCS) in 35 mm dishes. Colonies containing more than 20 cells were counted 12 days after plating.

RESULTS

We designed for the b2a2 transcripts expressed in human KYO-1 cells two double-stranded [siRNA(*dx1*) and siRNA(*dx2*)] and one single-stranded [siRNA(*AS-S4*)] siRNAs (Figure 1). The two double-stranded siRNAs have been synthesized with typical 3′-overhang deoxythymidine dimers (*14*). Their mRNA targets encompassed the b2a2 junction, and one was shifted three nucleotides aside with respect to the other. Additionally, we synthesized two control siRNA duplexes, siRNA(*c1*) and siRNA(*c2*), with 3 and 4 base-pair substitutions, respectively. Single-stranded siRNA(*AS-S4*) corresponded to the antisense (AS) strand of siRNA(*dx1*). To enhance its resistance to the endogenous nucleases, two contiguous phosphodiester groups at both strand termini were replaced with two phosphorothioates (*24*). To efficiently deliver the designed siRNAs into CML cells, we tested several liposome transfection reagents and also conducted electroporation experiments. We found by flow cytometry that FITC-labeled single- and duple-stranded siRNAs, complexed with oligofectamine, were taken up by more than 90% of the cell population, without significant reduction of cell viability (Figure 2A). When the FITC-labeled siRNAs were delivered without liposome, a lower fraction of cells (about 60%) appeared to have internalized the synthetic RNAs. To exclude the fact that the fluorescence associated to the cells was not due to the RNA molecules attached to the cell membranes, we analyzed the siRNA-treated KYO-1 cells by confocal laser microscopy. Figure 2B shows KYO-1 cells treated with FITC-labeled siRNA(*dx1*) in the presence (a−c) and absence (d−f) of oligofectamine. a and d show the nuclei stained in red with propidium iodide of siRNA-treated cells. b and e show the fluorescent light emitted by FITC-labeled siRNA(*dx1*), while c and f show the superimposition of a and b and d and e. When the images are taken together, they show that siRNA(*dx1*) is poorly taken up by KYO-1 cells in the absence of liposome, while with oligofectamine, siRNA(*dx1*) appears located mainly in the cytoplasm. Similar results were obtained with single-stranded FITC-labeled siRNA(*AS*) (not shown). We also delivered siRNAs to KYO-1 cells by electroporation and observed that with a double pulse (500 V, 25 μF, ∞ Ω) most of the cells imported the RNA molecules (not shown).

KYO-1 cells were treated with 100 nM anti-b2a2 and control siRNAs, and the level of *bcr*/*abl* mRNA was first measured by competitive RT-PCR. In brief, mRNA extracted from equal amounts of treated KYO-1 (expressing b2a2 transcripts, target) and untreated K562 (expressing b3a2

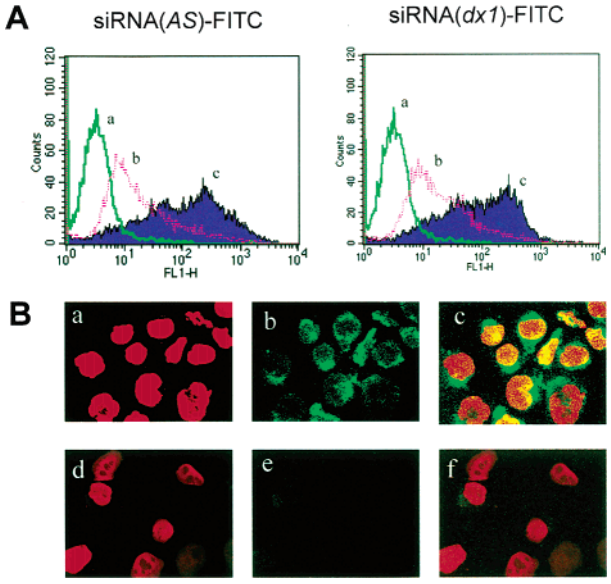

FIGURE 2: (A) FACS analysis of untreated and FITC-siRNA-treated KYO-1 cells. FITC-siRNA(*dx1*) and siRNA(*AS-S4*) (100 nM) were delivered to the cells with or without oligofectamine. The fluorescence associated to KYO-1 cells is plotted against the number of cells. Peak a, basal fluorescence associated to untreated cells; peak b, acquired fluorescence because of the FITC-labeled siRNA delivered to the cells without lipofection; and peak c, acquired fluorescence because of the FITC-labeled siRNA delivered to the cells with oligofectamine. (B) Confocal laser micrographs of KYO-1 cells treated for 5 h with FITC-labeled siRNA(*dx1*) in the presence of oligofectamine (a−c) and in the absence of oligofectamine (d−f) (a and d, propidium stained cells; b and e, fluorescence light emitted by FITC-labeled siRNAs; c and f, overlay of a and b and d and e).

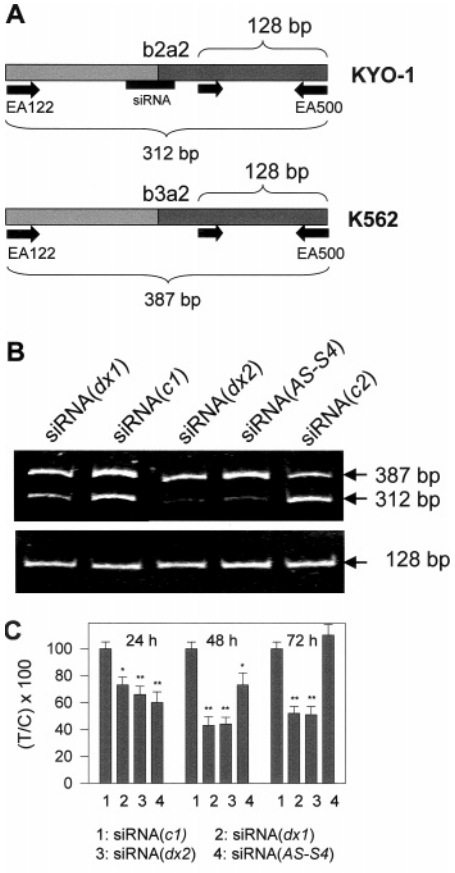

FIGURE 3: (A) Schematic representation of the competitive RT-PCR. mRNA from an equal number of untreated K562 (competitor) and siRNA-treated KYO-1 cells was extracted and subjected to RT-PCR. Using EA122 and EA500 primers, from K562 (b3a2 junction) and KYO-1 (b2a2 junction) cells, two amplified *bcr*/*abl* bands of 387 and 312 bp were obtained, respectively. When the KYO-1 cells were treated with siRNA(*dx1*), siRNA(*dx2*), or siRNa(*AS-S4*), the intensity of 312 bp but not that of 387 bp was significantly reduced, compared to the band obtained by treating KYO-1 cells with control siRNAs. (B) Typical competitive RT-PCR (24 h following transfection) showing that single- and double-stranded siRNA(*AS-S4*), siRNA(*dx1*), and siRNa(*dx2*) suppressed the level of *bcr*/*abl* mRNA in the treated KYO-1 cells. As the loading control, a 128-bp fragment from wild-type *abl* was amplified in each sample. (C) Histograms at 24, 48, and 72 h following transfection, showing the level of *bcr*/*abl* b2a2 transcripts in siRNA-treated KYO-1 cells. The ordinate reports the (T/C) × 100, where C is the 312/387 bp ratio in the control cells and T is the 312/387 bp ratio in siRNA-treated cells. The results shown are the means ± standard error (SE) of four independent experiments. A standard *t* test versus the control was performed (**, $p < 0.01$; *, $p < 0.05$)

transcripts, competitor) cells was transformed into cDNA and amplified by PCR. Because the target and competitor shared the same primer recognition sites, the amplification gave rise to one DNA fragment of 387 bp from K562 and one of 312 bp from KYO-1 cells, in virtue of their different junction (Figure 3A). The ratio between the 312 and 387 bp fragments provided an estimate of the amount of b2a2 transcripts relative to b3a2 transcripts. The efficiency of the PCR reaction was estimated by amplifying a 128-bp DNA fragment from wild-type *abl*. Figure 3B shows a typical RT-PCR experiment at 24 h following transfection, while Figure 3C shows a histogram summarizing the results obtained at 24, 48, and 72 h. Three observations can be done: (i) siRNA(*dx2*) suppresses b2a2 transcripts at an earlier time than siRNA(*dx1*); (ii) like siRNA(*dx1*), the single-stranded and thioated siRNA(*AS-S4*) activates the RNAi pathway; (iii) siRNA(*AS-S4*) catalyses a transcript suppression up to 48 h, whereas its double-stranded cognate siRNA(*dx1*) catalyses a longer-lasting effect (at least up to 72 h).

Next, we examined by immunoblotting the level of protein p210 $^{BCR/ABL}$ in treated KYO-1 cells. To this purpose, the level of p210$^{BCR/ABL}$ was measured at 55, 72, and 96 h following transfection (Figure 4). The percent treated (T)/untreated (C) ratio between p210$^{BCR/ABL}$ and β-actin in T and C KYO-1 cells are reported in the enclosed plot. It can be seen that 55 h following transfection siRNA(*dx2*) strongly suppressed p210$^{BCR/ABL}$, whereas siRNA(*dx1*) did not. Conversely, after post-transfection for 72 h, both anti-b2a2 RNA duplexes knocked down the *bcr*/*abl* gene to 20−30% of the control. Ninety-six hours post-transfection, i.e., after four cell divisions, the RNAi effect appeared reduced, although the effect promoted by siRNA(*dx2*) is still significant (b2a2 transcripts about 60% of the control). It is however clear that, over the three times considered, siRNA(*dx2*) exhibited a stronger capacity to inhibit *bcr*/*abl* than siRNA(*dx1*). The inhibition promoted by the two siRNAs was highly specific, because the level of expression of β-actin was not affected by anti-b2a2 or control siRNAs. To further test the specificity of the designed siRNAs, we transfected K562 cells, which express b3a2 transcripts, with the two anti-b2a2 siRNA duplexes. We found that, 72 h following transfection, *bcr*/*abl* was not inhibited, confirming that the designed siRNAs promoted a gene-silencing process highly specific for the b2a2 transcripts (Figure 5). In addition, we transfected KCl22

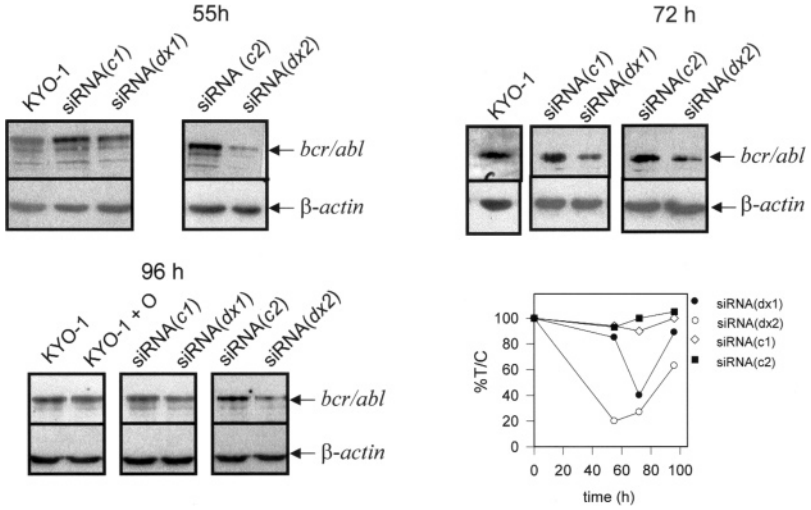

FIGURE 4: Western blot analysis showing at 55, 72, and 96 h following transfection the level of $p210^{BCR/ABL}$ in KYO-1 cells treated with 100 nM anti-b2a2 [siRNA(*dx1*) and siRNA(*dx2*)] or control [siRNA(*c1*) and siRNa(*c2*)] siRNAs. The siRNAs were delivered to the cells complexed with oligofectamine. As the loading and specificity control, we measured in each sample the level of β-actin. The plot shows the level of $p210^{BCR/ABL}$ expressed as % T/C, where T is the ratio between $p210^{BCR/ABL}$ and β-actin in siRNA-treated cells and C is the same ratio in untreated cells. Data uncertainty evaluated from two experiments is about 10−15%.

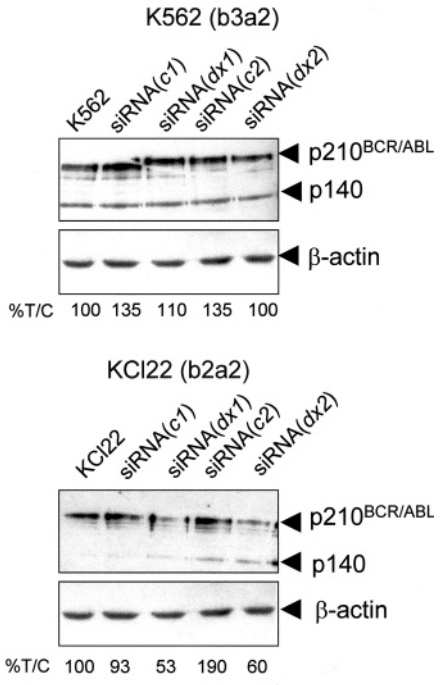

FIGURE 5: Level of $p210^{BCR/ABL}$ in K562 cells containing the b3a2 junction (above) and in KCl22 cells containing the b2a2 junction (below), 72 h following siRNA transfection. The numbers below each panel indicates the relative % T/C ratios between $p210^{BCR/ABL}$ and β-actin in T and C cells. Data uncertainty is about 10−15%.

cells, which by contrast express b2a2 transcripts, and found, as expected, that both siRNA(*dx1*) and siRNA(*dx2*) significantly reduced the level of protein $p210^{BCR/ABL}$ (Figure 5).

Because single-stranded siRNA is also able to trigger the RNAi process (*25*), we compared the ability of siRNA(*AS-S4*) and of its double-stranded cognate siRNA(*dx1*) to down-regulate *bcr*/*abl*. Considering that (i) single-stranded siRNA(*AS-S4*), although partially thioated, may undergo some nuclease degradation; (ii) siRNA(*AS-S4*) exhibits maximal activity on the mRNA level 24 h following transfection, as shown by RT-PCR; (iii) $p210^{BCR/ABL}$ has a long half-life [>40 h (*26*)], we delivered the siRNAs to KYO-1 cells by two

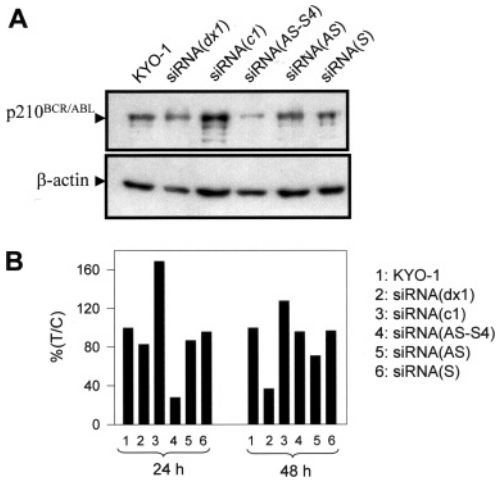

FIGURE 6: Western blot after two electroporations (the second 24 h after the first). (A) Protein levels 24 h following the second electroporation with single-stranded siRNA(*AS-S4*), double-stranded siRNA(*dx1*), and control siRNAs. (B) Histogram showing the level of $p210^{BCR/ABL}$ at 24 and 48 h following the second electroporation. Protein level is expressed in terms of % T/C, where T is the ratio between $p210^{BCR/ABL}$ and β-actin in treated KYO-1 cells and C is the ratio in untreated KYO-1 cells. Data uncertainty evaluated from two experiments is about 10−15%.

electroporation treatments. The level of $p210^{BCR/ABL}$, 24 h after the second electroporation, was reduced to 28% of the control by siRNA(*AS-S4*) and to 83% of the control by siRNA(*dx1*) (Figure 6A). It is noteworthy that the single-stranded oligoribonucleotide with all phosphodiester linkages, siRNA(*AS*), was almost not functional, probably because it is susceptible to nuclease degradation. The western analysis was repeated 48 h after the second electroporation and all of the results obtained are shown in the histogram of Figure 6B. It can be noted that siRNA(*AS-S4*) activates the suppression of *bcr*/*abl* at an earlier time than double-stranded siRNA(*dx1*), because the entry in the RNAi pathway of the former is facilitated with respect to the latter (*27*).

We then examined whether the depletion of $p210^{BCR/ABL}$ had any biological consequences on KYO-1 cells. We observed that the siRNA treatment had an effect on the cell

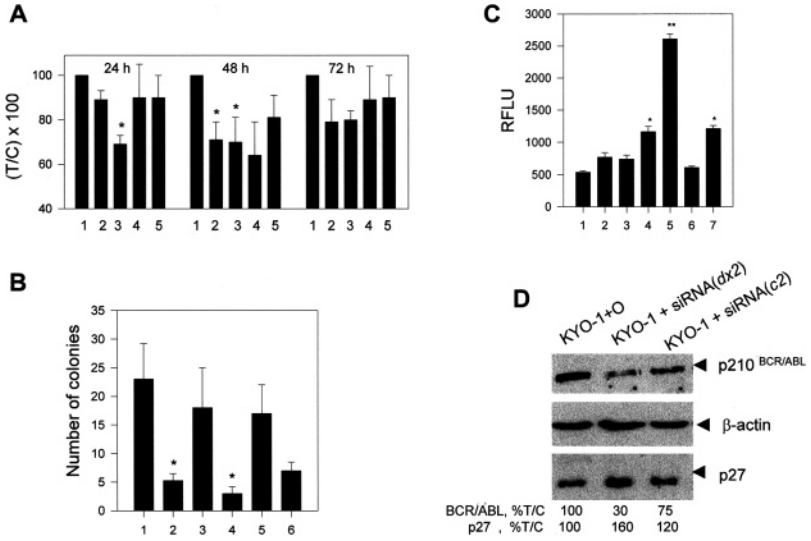

FIGURE 7: (A) Proliferation assay. 1: siRNA (c2); 2: siRNA(*dx1*); 3: siRNA(*dx2*); 4: siRNA(AS-S-4); 5: siRNA(c3). siRNA(*dx1*), siRNA(*dx2*) and siRNA(*AS-S4*) inhibits the proliferation of KYO-1 cells. The antiproliferative effect was measured by trypan blu counting of dead cells, at three post-transfection times (24, 48 and 72 h). siRNA(c3) is a single-stranded 20-mer oligonucleotide with a random sequence used as control; (B) Inhibition of colony formation in agar of KYO-1 cells 48 h post-transfection with siRNA(*dx1*) and siRNA-(*dx2*). 1: KYO-1 + oligofectamine; 2: KYO-1 + siRNA(*dx1*); 3: KYO-1 + siRNA(c1); 4: KYO-1 + siRNA(*dx2*); 5: KYO-1 + siRNA(c2); KYO-1 + siRNA(*AS-S4*); (C) Apoptose assay showing that a 48 h following transfection, siRNA(*As-S4*), siRNA(*dx1*) and siRNA(*dx2*) significantly enhance in KYO-1 cells the caspase activity. 1: KYO-1; 2: KYO-1 + oligofectamine; 3: KYO-1 + siRNA(c1); 4: KYO-1 + siRNA(*dx1*); 5: KYO-1 + siRNA(*dx2*); 6: KYO-1 + siRNA(c2); 7: KYO-1 + siRNA(*AS-S4*); (D) Western blots at 72 h post-transfection showing that downregulation of *bc*/*abl* by siRNA(*dx2*), results in upregulation of p27: a protein inhibitor of the cell cycle. The data shown in panels A, B and C are means ± SE of several independent experiments. A standard *t*-test versus control was performed (**: $P < 0.01$; *: $P < 0.05$).

viability. The percent ratio of viable cells between T and C cells is shown in the histogram of Figure 7A. Note that the anti-b2a2 siRNAs reduced the number of viable cells by 30−40% compared to the control, 48 h following transfection. The effect on growth appeared stronger when we measured the capacity of colony formation in agar of the KYO-1 cells, 48 h following treatment with the anti-b2a2 and control siRNAs. Counting the colonies with at least 20 cells, we found that both siRNA(*dx1*) and siRNA(*dx2*) reduced the number colonies 5-fold (Figure 7B). We then addressed the question whether the growth inhibition promoted by the designed siRNAs was due to apoptosis. To this purpose, we measured the caspase activity in KYO-1 cells, 48 h following treatment with siRNA(*dx1*), siRNA(*dx2*), siRNA(*AS-S4*), or control siRNA(*c1*) and siRNA(*c2*). Apoptosis was measured by the appearance of a fluorescent product generated by the caspases. Figure 7C shows that siRNA(*dx2*) induced a level of caspase activity that was about 2.5-fold higher than that observed with control siRNA(*c2*), suggesting that siRNA-(*dx2*) triggers apoptosis in KYO-1 cells. A lower caspase activity was induced by siRNA(*dx1*) and siRNA(*AS-S4*). Finally, Figure 7D shows that the down-regulation of *bcr*/*abl* by siRNA(*dx2*), the more efficient of the two double-stranded siRNAs used in this study, results in upregulation of protein p27, an inhibitor of protein cdk2 that regulates the entry into the cell cycle (*28*−*29*).

DISCUSSION

Small interfering RNAs offer a potent tool to analyze gene function and to downregulate the expression of disease-associated genes. In this study, we demonstrate that the leukaemic *bcr*/*abl*-fused gene can be specifically targeted by double- and single-stranded siRNAs, without affecting the expression of unrelated genes (including the housekeeping parental *abl* gene). We have performed our study using Philadelphia positive KYO-1 and KCl22 cells, which express a *bcr*/*abl* mRNA with the b2a2 junction, with the purpose of validating efficient siRNA targets to suppress the b2a2 transcripts. Although both double-stranded siRNA(*dx1*) and siRNA(*dx2*) were found to be able to knock down *bcr*/*abl*, the latter showed a significantly stronger silencing capacity than the former. This different behavior can be rationalized in terms of structural features of the double-stranded siRNAs. It has been shown that siRNA functionality strongly correlates with flexibility at the duplex end containing the 5′-AS sequence and with the internal stability across the 9−14-nucleotide region (*30*−*32*). The internal stabilities of pentameric subsequences measured from the 5′-AS end of both siRNA duplexes were determined using available nearest-neighbor stacking energies for duplex RNA (*33*). The data obtained are shown in Figure 8. It can be seen that the 5′-AS end of siRNA(*dx1*) (∼10 kcal/mol) is more stable, thus, less flexible, than the corresponding end in siRNA-(*dx2*) (∼7.4 kcal/mol) by 2.6 kcal/mol. The average internal stability across bases 9−14 is similar in both siRNA duplexes, 6.8 ± 0.4 kcal/mol. Khovorova et al. (*30*) and Ue-Tei et al., (*32*) showed that enhanced flexibility at the 5′-AS sequence strongly correlates with siRNA functionality, because siRNA, after binding to RISC, undergoes unwinding to form an active RISC−RNA complex. Because the 5′-AS sequence of siRNA(*dx2*) is less stable by 2.6 kcal/mol compared to that of siRNA(*dx1*), the formation of an active RISC−RNA complex is easier with siRNA(*dx2*) than with siRNA(*dx1*). This should explain why siRNA(*dx2*) showed a higher activity than siRNA(*dx1*). Although the internal stability across the 9−14 region seems to be important in

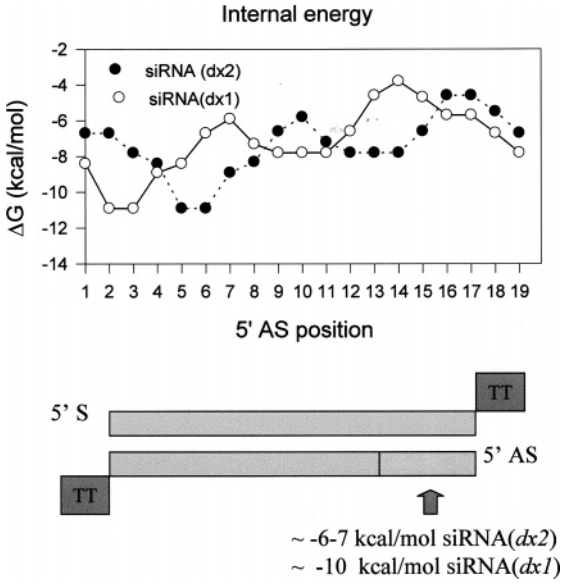

FIGURE 8: Internal stability profiles of siRNA(*dx1*) and siRNA-(*dx2*). Standard Gibbs free energies of siRNA(*dx1*) and siRNa-(*dx2*) duplexes, calculated according to available near-neighbor-stacking energy data. The pentamer subsequence of siRNA(*dx2*) containing the 5′-AS end is more flexible (−6−7 kcal/mol) than the pentamer subsequence of siRNA(*dx1*) (−10 kcal/mol).

the RNAi process, in this case, it is not discriminatory (*30*−*32*).

Single-stranded siRNA(*AS-S4*) is found to promote a short-lasting silencing effect compared to that triggered by its double-stranded cognate siRNA(*dx1*). This may correlate with the fact that siRNA(*AS-S4*), although partially thioated, may undergo some degradation by the endogenous nucleases (*24*). This is in accordance with the observation that the full phosphodiester analogue, siRNA(*AS*), which is expected to be more susceptible to nuclease degradation, was not found to be active.

In a previous study from this laboratory, we demonstrated that an AS PNA was able to suppress in KYO-1 cells the b2a2 transcripts to 35% of the control, 48 h following PNA treatment (10 μM) (*12*). In this study, we obtained a stronger suppression of b2a2 transcripts up to 72 h, by using a duplex RNA, siRNA(*dx2*), at a concentration 2 orders of magnitudes lower (100 nM). To our knowledge, this is the first report describing the validation of a strong RNAi target specific for the b2a2 transcripts expressed in leukaemic cells. In the literature, there are three studies on the site-directed targeting of *bcr*/*abl* mRNA by siRNAs, but all are specific for b3a2 transcripts (*18*−*20*). The inhibition effect reported is roughly similar to that find in this work (reduction of the protein level up 80%).

Previous studies have shown that the development of CML and the resistance of leukaemic cells to chemotherapy correlate with the potent antiapoptotic activity of p210$^{BCR/ABL}$ (*34*). Our data indicate that the down-regulation of *bcr*/*abl* by siRNA makes KYO-1 cells susceptible to apoptosis. In fact, siRNA(*dx2*) and, to a lesser extent, siRNA(*dx1*) and siRNA(*AS-S4*), induce higher levels of caspase activity with respect to the control, resulting in significant inhibition of cell growth and capacity of colony formation in agar. Another interesting aspect that emerged is that the suppression of *bcr*/*abl* results in up-regulation of p27, an inhibitor of cdk2, which is an essential cell-cycle kinase regulating the entry into the S phase (*28*−*29*).

The capacity to efficiently suppress the level of p210$^{BCR/ABL}$ has an enormous therapeutic significance. Since 1998, imatinib has been used in the treatment of leukaemia (*35*). This drug inactivates the tyrosine kinase activity of p210$^{BCR/ABL}$ and interrupts signal transmission to the nucleus (*20*). *In vitro* studies have shown that cells may develop resistance to imanitib, through *bcr*/*abl* amplification and/or overexpression (*36*). These observations suggested that imatinib used alone is less efficacious against CML or ALL than when used in conjunction with molecules that are able to down-regulate the expression of *bcr*/*abl*. In this context, anti-b3a2 and anti-b2a2 siRNAs may be promising drugs to be associated to imanitib therapy.

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