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Effects of siRNAs in combination with Gleevec on K-562 cell proliferation and Bcr-Abl expression

Benjamin E. Baker[1,2], Daniel P. Kestler[3] & Albert T. Ichiki[1,*]
[1]*Department of Medical Genetics, University of Tennessee Graduate School of Medicine, 1924 Alcoa Highway, Box U-2, Knoxville, TN, 37920, USA;* [2]*University of Tennessee College of Medicine, Memphis, TN, USA;* [3]*Human Immunology and Cancer Research Program, Department of Medicine, University of Tennessee Graduate School of Medicine, Knoxville, TN, USA*

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Summary

RNA interference (RNAi) is the repression of gene expression through a cellular mechanism of transcript-specific mRNA degradation. RNAi has been observed in human cells and applied to the modulation of a variety of human transcripts. Our goals were to deliver small interfering RNA (siRNA) using a liposome-based method, and to show Bcr-Abl siRNA specificity against K-562 cells, alone or in combination with Gleevec. Both synthetic (syn) siRNA, consisting of homogeneous 21-nucleotide-long RNA duplexes specific for the *Bcr-Abl* fusion site, and recombinant (r)-generated Bcr-Abl siRNA were employed. siRNA was transfected into K-562 cells with greater than 90% efficiency using RNAiFect™, as judged by fluorescence analysis. The Bcr-Abl transcript was inhibited using either siRNA preparation as measured by RT-PCR or real-time PCR. The IC_{50} of Gleevec in the K-562 subline F_1 was lowered over 3-fold from 0.2 to 0.06 μM in cells transfected with either syn or rBcr-Abl siRNA. No effect was observed in cells after transfection with an irrelevant control siRNA. Therefore, K-562 cells transfected with RNAifect deliver Bcr-Abl siRNA efficiently and the Bcr-Abl siRNA decreased the IC_{50} of Gleevec required to inhibit the high levels of Bcr-Abl protein found in K-562 cells.

Introduction

RNAi is an evolutionarily conserved pathway in eukaryotes that can target a gene, resulting in specific silencing through the identification and degradation of gene-specific mRNA transcripts [1]. The ability to target and interfere with specific RNA presents an approach to study gene modulation, screen for gene function, and intervene therapeutically in malignant and non-malignant pathologies. The optimal length of dsRNA for interference is 21 base pairs and the size is integral to the RNAi response initiated by the cell [2]. Since synthesis methods of siRNA have steadily improved, recent focus has been placed on the delivery of RNAi for both *in vitro* and *in vivo* applications. The Bcr-Abl translocation present in chronic myelogenous leukemia (CML) has been a target for RNAi studies and has been silenced using lipid-base amine liposome [3–5], electroporation [6, 7], and vector-based RNAi constructs [8, 9]. The K-562 cell line expresses high levels of the Bcr-Abl fusion transcript and expresses high amounts of Bcr-Abl protein, much greater than primary CML cells, which makes it a rigorous model for testing *in vitro* silencing of Bcr-Abl [10]. Imatinib mesylate (Gleevec) specifically inhibits the Bcr-Abl tyrosine kinase, decreases proliferation, enhances differentiation, and confers susceptibility to apoptosis, in

*To whom correspondence should be addressed. Fax: +1-865 544-9460; E-mail: aichiki@mc.utmck.edu

part through the down regulation of Bcl-X_L [11]. Chronic Gleevec treatment can result in the development of resistant clones; hence, interferon-α, etoposide, and daunorubicin have been administered with Gleevec in the treatment of CML [12]. In this study, siRNA was delivered using a liposomal delivery system and the effects of Bcr-Abl siRNA were examined alone and in combination with Gleevec on K-562 cells. Our results show that Bcr-Abl siRNA can modulate both the transcript and polypeptide synthesis and can lower the amount of Gleevec necessary to block proliferation of CML cells. These results corroborate the findings of other groups that siRNA has the potential to augment current treatment of CML.

Materials and methods

Cell culture and culture conditions

K-562 cells, subline F_1, were passaged weekly in Eagle's minimal essential medium supplemented with 15% fetal bovine serum (FBS) and non-essential amino acids, according to conditions previously described [13]. In brief, cells were recovered by centrifugation and suspended to 1×10^4 cells/ml in RPMI 1640 medium containing 10% FBS and 80 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C in 5% CO_2 in a humidified incubator.

Proliferation assay

K-562 cells were co-cultured with concentrations of Gleevec ranging from 0.05 to 1.6 μM (Gleevec was generously provided by Novartis, Basel, Switzerland). Proliferation was assayed daily for up to 4 days by assays determining the incorporation of [^{3}H]-thymidine into DNA. The method in brief, was to incubate 100 μl cells from the treated cultures with 0.25 μCi tritiated thymidine (ICN, Newport Beach, CA, USA) as previously described [14]. After 2 h of incubation, the cultures were harvested on Filtermats with a semiautomatic cell harvester. The radioactivity incorporated was expressed as counts per minute. All assays were performed in triplicate.

Proliferation was also determined by the colorimetric MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich) assay according to the methods of the manufacturer.

Recombinant siRNA preparation

The transcription plasmid Lit28i (New England Biolabs, Beverly, MA, USA) contained two opposing T7 promoter sites flanking the polylinker, allowing for *in vitro* dsRNA synthesis and was used to clone and transcribe the Bcr-Abl transcript region into dsRNA. The Bcr-Abl clone, containing a significant portion of the breakpoint region of the Bcr-Abl fusion gene (GenBank accession number M30829), was constructed by RT-PCR of K-562 cell RNA using a ThermoScript™ RT/PCR System (Invitrogen, Carlsbad, CA, USA) and a TaqMaster PCR kit (Eppendorf, Westbury, NY, USA). Bcr-Abl-specific primers used were: (sense) 5′-GGAGGGAGAACATCCGGGAGCA GC-3′ and (antisense) 5′-CCATTTTTGGTTGG GCTTCACAG-3′ (Sigma-Genosys, Woodlands, TX, USA). The 450 bp Bcr-Abl PCR product was isolated after agarose gel electrophoresis, purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), cloned into Lit28i at the EcoRV site, and sequenced for verification (Figure 1C). Subsequently, T7 polymerase-synthesized dsRNA was generated using an Ambion Silencer siRNA Cocktail kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The recombinant Bcr-Abl (rBcr-Abl)-specific dsRNA was digested with RNase III (Ambion) at 37 °C for 1 h, column purified and concentrated according to the manufacturer's protocol. Synthetic-Bcr-Abl-specific siRNA (synBcr-Abl) was purchased from Qiagen (Valencia, CA, USA).

Transfection

K-562 transfections were done at a concentration of 5.0×10^5 cells/ml in a volume of 0.2 ml. Transfections, using RNAiFect™ liposome delivery system (Qiagen) in accordance to the manufacturer's protocol, were performed with the following siRNAs: (1) irrelevant or non-silencing siRNA specific for *E. coli* maltose-binding protein gene (New England Biolabs); (2) synBcr-Abl siRNA; and (3) rBcr-Abl siRNA. In brief, cells were transfected in 24-well plates for 4 h in 10% FBS-containing RPMI 1640 medium and then diluted 10-fold into fresh

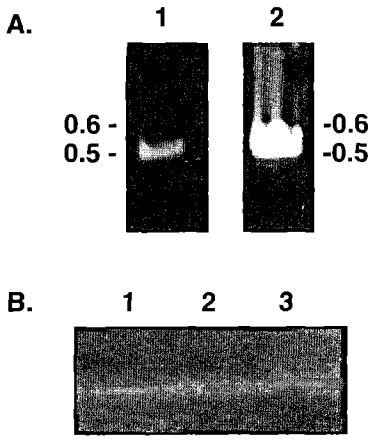

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Figure 1. Gel analysis of Bcr-Abl rRNA. Full (A) and diced (B) species. (A) (1) Bcr-Abl cDNA insert derived from K-562 cells by RT-PCR and cloning, (2) dsRNA product synthesized by runoff transcription of the Litmus Bcr-Abl clone. (B) Lanes: (1) 19-mer dsDNA oligo; (2) and (3). Product of two separate RNase III-digestions of recombinant Bcr-Abl dsRNA. (C) Nucleotide sequence of Bcr-Abl region delineating (underlined) the region of the synthetic siRNA. The Abl portion of the region is shown in bold following the arrow (⇓) insertion.

medium. Transfection concentration was at 1.6 μM siRNA. Transfection efficiency was measured using a non-silencing fluorescein-conjugated siRNA as analyzed by flow cytometry. The efficiency of transfection was repeatedly found to be greater than 90%. In order to detect decreases in the Bcr-Abl and Bcl-X_L protein levels, two transfections were required with the second transfection performed 24 h after the first.

Gene silencing analysis

Two-step reverse transcription PCR was performed with Superscript III (Invitrogen) according to the manufacturer's protocol and the subsequent PCR reactions were performed using an Eppendorf Taqmaster system according to the manufacturer' protocol. One-step real-time PCR was performed with the Gene Expression Assay system (Qiagen) using QuantiTect Bcr-Abl primer set (Qiagen) on the DNA Engine Opticon real-time system (MJ Research, Waltham, MA, USA).

Western blotting and immunoprecipitation

Total cell protein was isolated using cell lysis buffer containing 50 mM Tris–HCl, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl supplemented with 1 mM leupeptin, 1 mM $NaVO_4$, 1 mM NaF, 1 mM PMSF, and 1 mM peptide protease inhibitor cocktail (Sigma-Aldrich). Protein was resolved on SDS-denaturing polyacrylamide gels, electrophoresed onto nitrocellulose membranes, and subjected to Western analysis using established methods [15]. Antibodies used were obtained from the following sources: Bcr-Abl and Bcr (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Bcl-X_L (Cell Signaling, Beverly, MA, USA). Synthesis of nascent Bcr-Abl protein was determined after incubating the cells in cysteine/methionine (cys/met)-depleted medium (Sigma-Aldrich) for 30 min, then adding [^{35}S] cys/met, 50.0 μCi/ml (specific activity 1000 ci/mM) (ICN, Costa Mesa, CA, USA) and incubating the cells an additional 2 h [16]. Total protein was then

isolated, immunoprecipitated with Bcr-Abl-specific antibodies, gel electrophoresed and visualized after gel fluorography at −70 °C and film development using Kodak X-OMAT AR film (Kodak, Rochester, NY, USA).

Results

Bcr-Abl siRNA preparations yield Bcr-Abl-specific interference in K-562 cells

RNAi effects were measured using the following three approaches: (1) serial dilution of the RNA in RT-PCR, (2) real-time PCR and (3) Western and immunoprecipitation analysis of protein modulation. This approach provided evidence of modulation of both the Bcr-Abl mRNA and protein. Gel analysis of the run off rBcr-Abl dsRNA before and after RNaseIII treatment, which yielded a band of approximately 20–21 nucleotides, are shown in Figure 1A and B, respectively. The sequence of the rBcr-Abl dsRNA is shown in Figure 1C.

Synthetic and recombinant Bcr-Abl-specific siRNA preparations proved effective at suppressing Bcr-Abl gene expression as measured by both gel analysis of RT-PCR products and real time RT-PCR (Figure 2A and B). The RNAi effect is visible after gel analysis with 100 ng total K-562 RNA template in the RT-PCR assays of cells treated for 72 h. Both recombinant and synthetic Bcr-Abl siRNA-treated cells contained less Bcr-Abl RT-PCR product than the untreated control cells. When compared to the irrelevant and the untreated control cells (Figure 2A), both the synthetic and the recombinant Bcr-Abl-specific siRNA-treated cells showed a lower level of Bcr-Abl transcript, while no change in the control (aldolase) transcript was observed. Both Bcr-Abl siRNAs resulted in a near 60% reduction of the Bcr-Abl transcript, as measured through real time RT-PCR (Figure 2B). The GAPDH control showed an insignificant difference in the Bcr-Abl siRNA-transfected cells compared to the untreated and the RNAifect negative control (Figure 2C).

Detection of the Bcr-Abl protein modulation by siRNA proved difficult, which could be due to the long half-life of the polypeptide, high gene copy, and/or significant transcript numbers found in K-562 cells [10]. Two transfections were reported to be required to down-regulate the Bcr-Abl protein in K-562 cells [6]. Western blot analysis showed that synBcr-Abl siRNA lowered the Bcr-Abl protein compared to the irrelevant siRNA control (Figure 3A). This reduction was not observed with the rBcr-Abl siRNA preparation. To measure nascent protein, we pulsed cells with ^{35}S cys/met. After labeling and immunoprecipitation, the ^{35}S-Bcr-Abl protein production was found to be reduced in cells treated with the Bcr-Abl-specific siRNA preparations (Figure 3B). Further, Western analysis showed that the synBcr-Abl siRNA down-regulated the anti-apoptotic Bcl-X_L protein significantly compared to an irrelevant control siRNA (Figure 3A). This supports the observed relationship between Bcr-Abl and Bcl-X_L expression [17, 18].

IC_{50} of Gleevec

The IC_{50} of Gleevec was determined to be 0.2 μM by dose-response analysis using [^{3}H]-thymidine uptake, which was consistent with published IC_{50} values of Gleevec, (Figure 4A) [19].

RNAi and Gleevec in combination decreased proliferation

Proliferation of the K-562 cells transfected with the syn- or r-Bcr-Abl siRNAs showed a statistically significant decrease ($p < 0.01$ by analysis of variance) in proliferation when treated with 0.10 μM Gleevec for 72 h, as compared to the cells transfected with the irrelevant siRNA. The results from ^{3}H-thymidine, Figure 4B and the MTT assay Figure 4C were similar. No differences were observed at high levels of Gleevec, which are well above the 50% level of proliferation. SynBcr-Abl siRNA, (1.6 μM), alone showed a 15–20% inhibitory effect.

Discussion

There are increasing numbers of *in vitro* and *in vivo* reports where specific genes are silenced by RNAi. Treatments with siRNA, alone or in combination with drugs, could be effective as therapeutic agents in the future. Our results suggest that silencing the Bcr-Abl oncogene could lower the effective Gleevec dosage, reducing the possibility of developing

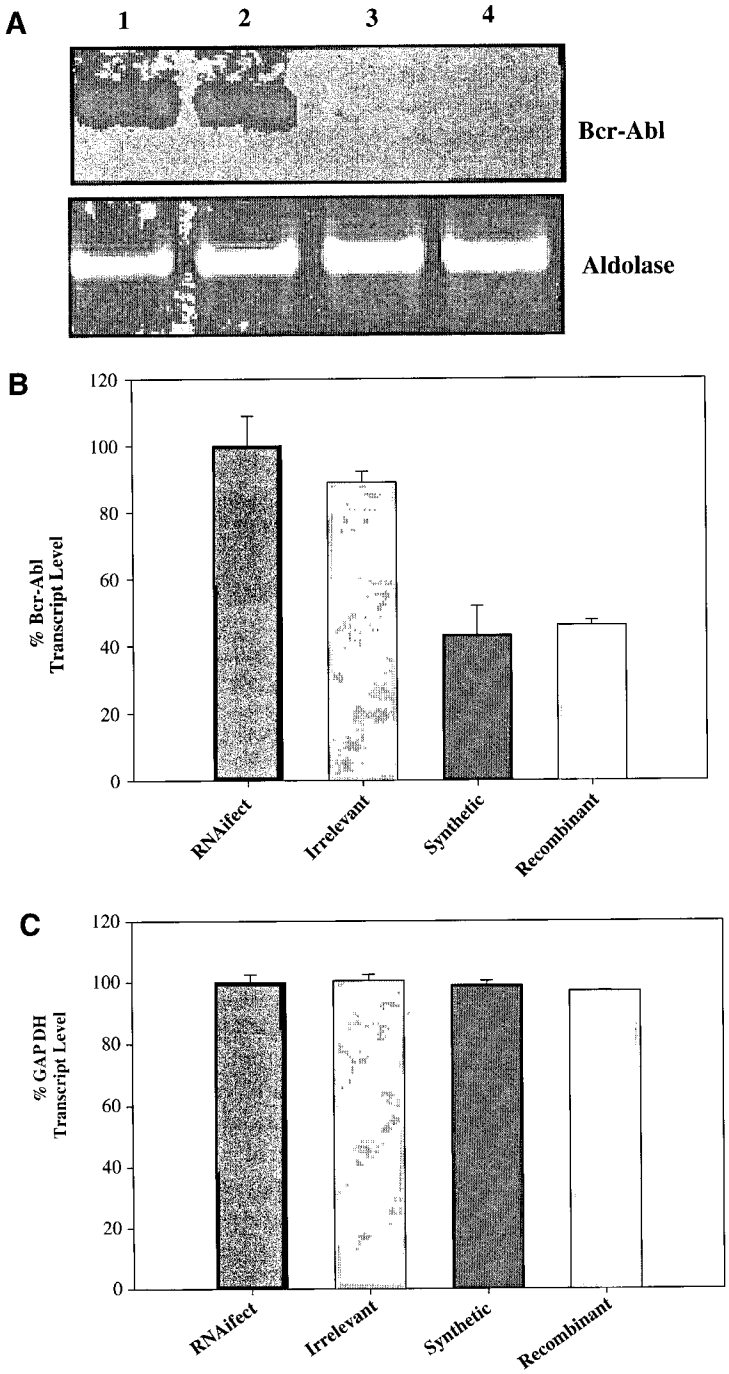

Figure 2. RNA-interference analysis of Bcr-Abl in K-562 cells by direct gel analysis and real-time PCR. (A) Gel analysis of Bcr-Abl RT-PCR of total K-562 RNA at 72 h post siRNA treatment with: (1) no treatment, (2) irrelevant siRNA treatment, (3) syn-Bcr-Abl siRNA, and (4) rBcr-Abl siRNA. The siRNA treatments had no effects on aldolase-specific RT-PCR reactions. (B) Real time RCR analysis of Bcr-Abl mRNA levels at 48 h after RNAi treatment. Transcript levels were normalized to the RNAifect-treated cells. The Bcr-Abl siRNA transcript levels for cells treated with either syn- or r- Bcr-Abl siRNA were approximately 2-fold lower than those of control treatments. Samples were assayed in quadruplicate and mean and standard deviations are presented in the graph. (C) The irrelevant or GAPDH mRNA transcript levels showed no effects by siRNA treatments of K-562 cells.

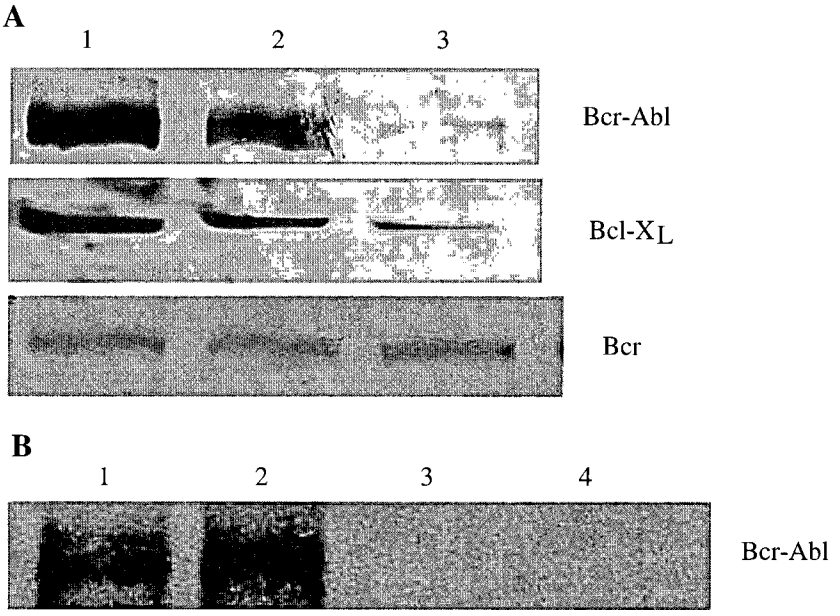

Figure 3. Western and nascent-synthesis analysis of Bcr-Abl protein after siRNA treatments. (A) Western analysis of Bcr-Abl, Bcl-X_L and Bcr expression in K-562 cells treated with (1) RNAifect, (2) irrelevant siRNA, and (3) synBcr-Abl siRNA. Bcr-Abl transcript appeared lowered in cells treated with synBcr-Abl siRNA (see lane 3), while Bcr-expression was not affected. (B) Immunoprecipitation of Bcr-Abl from cells metabolically labeled with [^{35}S]-cys/met. Immunoanalyses were of labeled cells previously treated with: (1) RNAifect-only, (2) irrelevant siRNA, (3) synBcr-Abl siRNA, and (4) rBcr-Abl siRNA. Both syn- and r-siRNA treatments showed a significant reduction in synthesis of nascent Bcr-Abl protein.

Gleevec-resistant mutants and, perhaps, extending the efficacy period of the drug in the CML patient. We demonstrated that it was possible to deliver both syn- and r-siRNA effectively using RNAifect, a lipid-based reagent, to transfect the K-562 cells with up to 90% efficiency and minimal loss of cell viability. The transfection procedure was performed in the presence of serum, minimizing the stress on the cell since other siRNA transfection methods require serum-free procedures. The high efficacy obtained suggests that siRNA could be delivered *in vivo* with suitable reagents, such as RNAifect, in combination with Gleevec treatment in parallel to viral or plasmid transfection delivery systems. A receptor-mediated liposomal delivery system was shown to deliver vascular endothelial cell growth factor RNAi that inhibited ocular herpetic stromal keratitis in mice [20].

While proliferation of the K-562 cells transfected with the two different preparations of Bcr-Abl siRNA was reduced, the synBcr-Abl siRNA centered around the break-point was more effective than the recombinant siRNA preparation at each Gleevec concentration assayed after 72 h of incubation. These differences may be due to the higher molar concentration of the effective synBcr-Abl breakpoint-specific dsRNA oligomer than effective products obtained from RNase III cleavage of the 450 bp rBcr-Abl dsRNA. The synBcr-Abl-siRNA preparation resulted in a significant reduction of Bcr-Abl mRNA as measured by real time analysis. Our premise was that a 300 bp recombinant siRNA template contained more Bcr-Abl siRNA species and would result in a greater reduction of the Bcr-Abl mRNA than that of a uniform, single synBcr-Abl siRNA. However, our results using either preparation yielded a similar inhibition of Bcr-Abl mRNA. This suggests that the number of unique Bcr-Abl interfering motifs are limited and, perhaps, localized. Down-regulation of the Bcr-Abl mRNA and protein required multiple transfections, as previously reported [6], and, as suggested, was likely due to siRNA dilution through cell divisions and the long half-life of the Bcr-Abl protein (>48 h). No detectable decrease of the Bcr-Abl protein was found in cells transfected with the rBcr-Abl siRNA preparations. As suggested above, this could reflect a lower concentration of specific Bcr-Abl "active" siRNA 21–23 bp as found in homogeneous

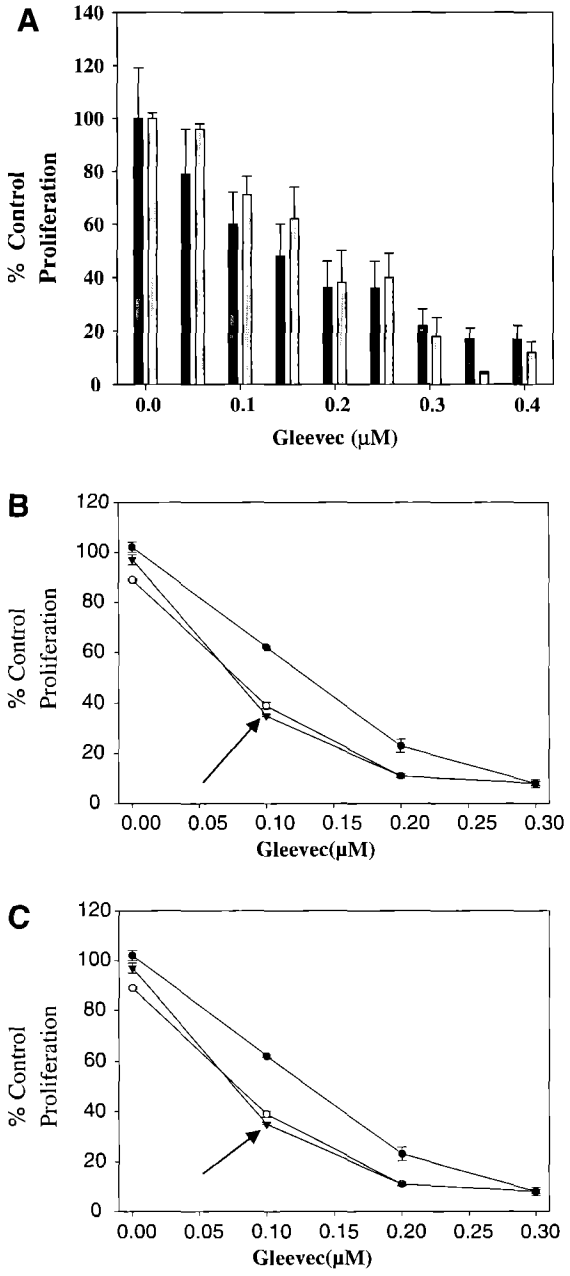

Figure 4. Dose response effects of Gleevec on proliferation by the incorporation of [^{3}H]-thymidine by K-562 cells normalized to the cpm incorporation of untreated cells (A). Analyses were done at 48 (black) and 72 (gray) h. Mean values with standard deviations are shown. Samples were measured in triplicate and experiments repeated three times. Combined effects of Bcr-Abl siRNAs and Gleevec on proliferation in K-562 cells were determined by ^{3}H-thymidine uptake (B) and MTT (C). Effects of syn- (open circle), r- (closed triangle) Bcr-Abl siRNA and irrelevant siRNA (closed circle) with varying doses of Gleevec for 72 h. All assays were done in quadruplicate and the experiments were repeated two times. With no Gleevec (o), there was a 15–20% inhibition with Bcr-Abl siRNA. Arrow indicated statistical lower proliferation by analysis of variance, $p < 0.01$, when the K-562 cells were treated with syn- or r-Bcr-Abl siRNA in combination with 0.10 μM Gleevec as compared to the irrelevant siRNA. Standard deviation of individual points was less than 5%.

synthesized siRNA, which did show the direct Bcr-Abl protein inhibition by Western analysis. In addition, the protein detected by Western analysis was most likely remnant Bcr-Abl and, therefore, production of Bcr-Abl protein was not being directly measured. Comparison of the efficacy of the syn- and r-Bcr-Abl-specific siRNA demonstrated that high copies of Bcr-Abl in the K-562 cells required the delivery of a higher level of siRNA as compared to cells with lower copies of Bcr-Abl, such as most primary CML cells.

Both siRNA preparations appeared effective in blocking the synthesis of nascent Bcr-Abl protein as analyzed by immunoprecipitation of lysates from ^{35}S-labeled cys/met pulse labeling of K-562 cells. SynBcr-Abl siRNA also decreased the level of Bcl-X_L, a downstream target for Bcr-Abl, as measured by Western analysis (Figure 3A). This suggests that the Bcr-Abl anti-apoptotic pathway had been abrogated. Determination of Bcr-Abl protein modulation by Western analysis is difficult in K-562 cells since the protein has been shown to have a long half-life [3, 6–7]. Thus, the ability to visualize significant modulation of Bcr-Abl polypeptide could be buffered by the longevity of the Bcr-Abl protein in K-562 cells.

The use of RNAi to modulate Bcr-Abl expression in CML cells and Bcr-Abl-expressing cell lines suggests a therapeutic role for such treatment. Many of the Bcr-Abl siRNA *in vitro* studies have employed vector-based siRNA delivery systems, which target cells delivering either plasmid or viral-based vector RNAi construct for cell expression of siRNA. Through the use of liposomal agents, siRNAs can be added directly to the cell culture or, perhaps, *in vivo* requiring no plasmid or viral vector transfection procedures and subsequent siRNA synthesis [20]. Recent findings showed that all Bcr-Abl can be down regulated in all common p210 and p190 Bcr-Abl variants using RNA interference [21]. By optimizing liposomal methods for siRNA delivery, such a procedure could be clinically relevant as a treatment.

In our study, K-562 cells had increased sensitivity to Gleevec in the presence of Bcr-Abl siRNA. This increased sensitivity has also been observed by others in cells expressing TEL-PDGβR fusion of the CMML-associated tyrosine kinase affected by Gleevec in the presence of PDGFβR-specific siRNA [22]. In the future, it could be important to employ a strategy of Gleevec treatment in combination with RNA interference in select genes of proliferation and apoptosis. In support of this rationale, K-562 cells treated with siRNA directed against Bcl-X_L or WT1 [23, 24] were observed to modulate apoptosis, cell proliferation and specific genes associated with these processes.

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