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Polymeric siRNA delivery targeting integrin-β1 could reduce interactions of leukemic cells with bone marrow microenvironment

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ABSTRACT

Uncontrolled proliferation of the myeloid cells due to *BCR-ABL* fusion has been successfully treated with tyrosine kinase inhibitors (TKIs), which improved the survival rate of Chronic Myeloid Leukemia (CML) patients. However, due to interactions of CML cells with bone marrow microenvironment, sub-populations of CML cells could become resistant to TKI treatment. Since integrins are major cell surface molecules involved in such interactions, the potential of silencing integrin-β1 on CML cell line K562 cells was explored using short interfering RNA (siRNA) delivered through lipid-modified polyethyleneimine (PEI) polymers. Reduction of integrin-β1 in K562 cells decreased cell adhesion towards human bone marrow stromal cells and to fibronectin, a major extracellular matrix protein for which integrin-β1 is a primary receptor. Interaction of K562 cells with fibronectin decreased the sensitivity of the cells to BCR-ABL siRNA treatment, but a combinational treatment with integrin-β1 and BCR-ABL siRNAs significantly reduced colony forming ability of the cells. Moreover, integrin-β1 silencing enhanced the detachment of K562 cells from hBMSC samples (2 out of 4 samples), which could make them more susceptible to TKIs. Therefore, the polymeric-siRNA delivery targeting integrin-β1 could be beneficial to reduce interactions with bone marrow microenvironment, aiding in the response of CML cells to therapeutic treatment.

1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by uncontrolled expansion of a select population of pluripotent hematopoietic progenitor cells at the expense of normal hematopoietic progenitor cells, resulting in abnormally high accumulation of ill-formed myeloid cells [1]. The hallmark of CML is the reciprocal chromosomal translocation of the chromosome 9 having *ABL* gene (a human homologue of *v-abl* oncogene carried by Abelson murine leukemia virus) and chromosome 22 that breaks in a specific breakpoint cluster region (BCR) and results in the formation of fusion oncogene *BCR-ABL*. The resultant protein has enhanced tyrosine kinase activity and stimulates multiple undesirable events, including uncontrolled proliferation, growth factor independence, decreased attachment to stroma cells and extracellular matrix along with reduced apoptosis [1,2]. The disease progression in CML can develop in three phases when left untreated; (i) the initial *chronic phase* that last from 3 to 6 years and where most patients are diagnosed, (ii) the subsequent *accelerated phase* (6-9 months), and (iii) the terminal *blast crisis phase* that lasts 3 to 6 months [3]. The presence of *BCR-ABL* fusion protein in all CML patients make it a unique, and highly viable therapeutic target. This fusion oncogene has been effectively controlled by the prototypical tyrosine kinase inhibitor (TKI) imatinib mesylate (IM). The IM binds to the amino acids present at the ATP binding site of BCR-ABL tyrosine kinase protein and stabilizes it in an inactive form. This eventually "switches-off" the downstream signaling pathways leading to leukemogenesis. The IM has become the front line drug to treat newly diagnosed CML and aims to maintain remission, prevent relapse and disease progression to the accelerated phase or blast crisis [4–6]. However, after the initial IM treatment, some patients become unresponsive to the drug due to point mutations at the IM-binding site of *BCR-ABL* and this led to development of second generation (such as dasatinib, nilotinib and bosutinib) and third generation TKIs (ponatinib) [7–9]. In addition to mutagenic response to IM, certain populations of CML cells become unresponsive to TKI treatment due to their direct interaction and binding with the bone marrow (BM) and/or extracellular matrix (ECM) proteins through cell surface molecules [10–12].

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Integrins are key cell-surface molecules that can regulate various intracellular signaling pathways such as proliferation, migration and apoptosis of the cells [13]. There are 24 different integrin receptors that are formed by dimerization of 18 α-integrin and 8 β-integrin subunits and their expression vary significantly from cell to cell [14]. The binding of CML cells to the ECM protein fibronectin through cell surface integrins was shown to induce drug resistance and provide protection from apoptosis by the activation of tyrosine kinases [13,15]. *BCR-ABL* expression was shown to alter Integrin-β1 (ITGB1) functioning in different studies; it leads to integrin-β1 clustering, cytoskeleton remodelling and increased binding to fibronectin and stromal cells [16,17]. The direct interaction of CML cells with stromal microenvironment play an important role in the drug resistance in CML patients and was demonstrated by the interaction of the CML model K562 cells with stromal cells which provided protection against apoptosis induced by IM treatment. The binding to bone ECM via integrins could activate various intracellular signaling pathways altering the expression of multiple genes as well as the secretion of various growth factors from the stromal environment, which could confer drug resistance and act as a sanctuary for minimal residual disease (MRD) [18,19]. In all these studies, the binding of CML cells was the key in providing resistance to TKI treatment and, if this binding event could be interfered, the sensitivity of CML cells to the drugs could be improved.

In this study, we hypothesize that the knockdown of a specific integrin molecule (integrin-β1) present on CML cells will improve the sensitivity of the cells to the TKI treatment. For this purpose, we used the well-established CML model K562 cells *in vitro*. This integrin knockdown was achieved using the siRNA approach, which has the advantage to target the specific genes of interest without altering other related genes. The effectiveness of siRNA-mediated silencing, however, depends on the efficiency of the gene delivery vector used. Polyethyleneimine (PEI) polymers are safe, non-viral delivery systems that can complex with siRNA to form nanoparticle complexes suitable for cell uptake. Lipid modification of PEI was shown to improve the interaction of cationic PEI/siRNA complexes with the anionic cell membrane. For this purpose, lipid-modified 1.2 kDa PEI (1.2PEI) polymers were utilized in this study to deliver dicer-substrate siRNA that helps to better incorporate into the RISC complex and provides higher knockdown as observed in our previous studies [20,21]. Here, we show that; (i) CML K562 cells can bind to fibronectin, (ii) integrin-β1 is highly expressed by K562 cells compared to other integrins, (iii) successful knockdown of integrin-β1 can reduce its binding of K562 cells towards fibronectin and human bone marrow stromal cells (hBMSC) and (iv) silencing of integrin-β1 can also help in the detachment of the leukemic cells attached to the stroma cells which can be targeted by TKIs. We also demonstrate that K562 cells can become resistant to BCR-ABL siRNA treatment when cultured on fibronectin and the knockdown of integrin-β1 improve the sensitivity to siRNA treatment. These are promising results to overcome drug resistance in CML and can form the basis of further evaluation in a preclinical animal model.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) with D-glucose, L-glutamine and sodium pyruvate, Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine and HEPES, DMEM/F12 with L-glutamine and HEPES, Hank's Balanced Salt Solution (HBSS), Penicillin-Streptomycin (10,000 U/mL), UltraPure DNase/RNase-free dH_2O, MEM Non-essential amino acids, GlutaMAX-I, TRIzol Reagent, chloroform, acetic acid, Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX were purchased from Thermo Fisher Scientific (Ottawa, Canada). Fibronectin, formaldehyde solution, methyl cellulose, thiazolyl blue tetrazolium bromide (MTT) and the primers used for various integrin gene expression analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethyleneimine of 1200 Da (1.2 PEI) was from Polysciences (Warrington, Pennsylvania). Fetal bovine serum (FBS) was from VMR Life Science Seradigm (Mississauga, Ontario). Phycoerythrin(PE)-labeled mouse anti-human CD29 (integrin-β1) was from BD Biosciences (Oakville, Canada). Dicer substrate siRNA (ITGB1 sense: 5'-AGUUAACAGUGAAGACAUGGAUGCT-3', antisense: 5'-AGCAUCCAUGUCUUCACUGUUAACUUC-3'), 6-carboxyfluorescein (FAM) labelled scrambled siRNA and a custom synthesized BCR-ABL siRNA (sense: 5'-GCAGAGUUCAAAAGCCCTT-3'. antisense: 5'-GGGCUUUUGAACUCUGCTT-3') were from IDT (Coralville, USA). Crystal violet from Allied Chemical. K562 cells and MDA-MB-231 cells were authenticated by genetic sequencing at the Centre for Applied Genomics, Toronto, Canada. TKI inhibitors Imatinib and Dasatinib were kindly provided by Dr. Xiaoyan Jiang (Terry Fox Laboratory, British Columbia Cancer Agency and Department of Medical Genetics, University of British Columbia).

2.2. Cell models and culture

The *BCR-ABL* positive K562-WT (wild type) cell line established from a CML patient in blast crisis was purchased from the American Type Culture Collection (Virginia, USA) and K562 cells expressing green fluorescent protein (GFP) were maintained in RPMI medium containing 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2. Spent medium was removed every third day by centrifugation at 500 rpm (5 min) and diluted 10 times in 20 mL of fresh medium for cell expansion. MDA-MB-231 breast cancer cell line was kindly provided by Dr. Judith Hugh (Faculty of Medicine and Dentistry, University of Alberta, Edmonton) and were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2. After reaching 80% confluency, MDA-MB-231 cells were passaged using 0.05% Trypsin/EDTA at 37°C for 2 min and complete DMEM was used to collect the cells followed by centrifugation at 600 rpm (5 min). MDA-MB-231 cells were seeded 24 hours prior to the treatment. Human bone marrow stromal cells (hBMSCs) were isolated from patients (between 25-50 years of age) based on a procedure [22] approved by the Research Ethics Board of University of Alberta. These hBMSC cells were maintained in DMEM/F12 with 12% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1% MEM Non-essential amino acids and 0.1% GlutaMAX-I at 37°C and 5% CO_2.

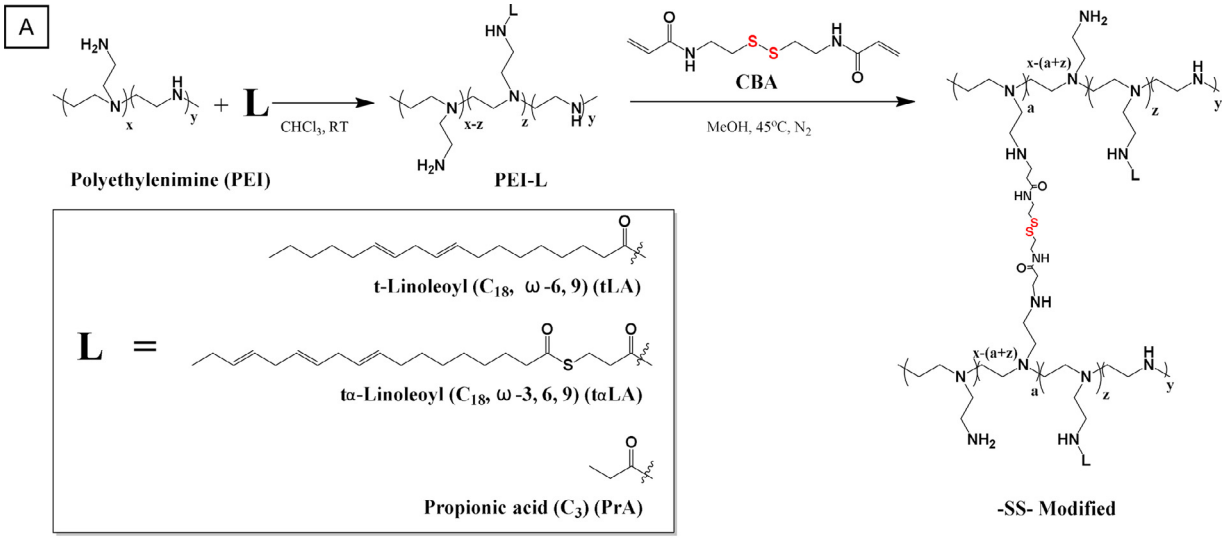
2.3. Preparation of polymers and polymer/siRNA complexes

A library of lipid-modified 1.2PEI polymers was synthesized through amide and thioester linkages using linoleic acid (LA), α-linoleic acid (αLA) and propionic acid (PrA) based on previously published protocols [23–26]. A redox-sensitive polymer library was also prepared through disulfide cross-linking (-SS-) of lipid-modified PEIs via condensation reaction using cystamine bisacrylamide (CBA) as the cross-linker [24] (Figure 1A). In all cases, the degree of lipid substitution on the polymers was determined through ^{1}H-NMR spectroscopy as described earlier [23-26]. Figure 1B summarizes the polymers explored in this study.

The polymer/siRNA complexes (typically at 9:1, w/w) were prepared in serum-free media (RPMI for K562 and DMEM for MDA-MB-231 cells) by incubating the desired polymer with siRNA for 30 min at room temperature. Lipofectamine™ 2000/siRNA complexes (2:1, w/w) (for Figure 4C, and Figure S3) and Lipofectamine™ RNAiMAX/siRNA (5:1, w/w) (for Figure 3, 4B and S4) were prepared according to the optimized protocol provided by the manufacturer.

2.4. Fibronectin binding assay

The fibronectin binding assay was carried out based on a previously published protocol [27] with minor modifications. Briefly, 40 µL of 25 µg/mL concentration of fibronectin was coated onto 96-well flat bottom



B

S.No	Unmodified	-SS- Modified
1	1.2PEI	1.2PEI-ss
2	1.2PEI-tLA2	1.2PEI-tLA2-ss
3	1.2PEI-tLA6	1.2PEI-tLA6-ss
4	1.2PEI-tαLA2	1.2PEI-tαLA2-ss
5	1.2PEI-tαLA6	1.2PEI-tαLA6-ss
6	1.2PEI-PrA1	1.2PEI-PrA1-ss

Fig. 1. (A) Schematic representation of polymer synthesis by grafting lipids *via N*-acylation followed by disulfide cross-linking (-SS- Modified). Lipid grafted PEI polymers were reacted with cystamine bisacrylamide (CBA) at 45^{0}C in methanol under N_2 environment to prepare the disulfide cross-linked polymers. (B) List of polymers explored in this study. The lipid substituted polymers (unmodified) and its corresponding disulfide cross-linked polymers (-SS- Modified).

plates at room temperature for 1 hour. Viable cells were counted using trypan blue dye exclusion assay and equal number of cells were added in triplicates to the fibronectin coated plates. Following 1 hour of incubation at 37°C, plates were inverted and further incubated for 3, 7, 19 and 23 hours at 37°C. Cells were fixed with 3.7% formaldehyde followed by staining with 0.1% crystal violet (in water) for 30 min and washed twice with HBSS to remove excess dye. The cell-associated dye was solubilized with 10% acetic acid and the absorbances were measured at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments). Microscopic images were also captured at different time points. For the integrin-β1 silencing studies, the cells were treated with desired concentrations (see Figure legends) of polymer/siRNA complexes for 3 days, following which K562 cells were collected, centrifuged at 1400 rpm for 5 min and resuspended in 100 µl of HBSS. Cells were stained with DiI dye for 20 min at room temperature and excess dye was removed by HBSS wash (twice). Cells were then re-suspended in desired volume of fresh complete medium (10% FBS) and fibronectin binding was carried out for 4 hours. The non-adherent cells were removed by washing and the fluorescence was recorded at excitation of 536 nm and emission of 607 nm using Fluoroskan Ascent plate reader (Thermolab Systems).

2.5. Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

The expression levels of integrins α2b (ITGA2B), α3 (ITGA3), α5 (ITGA5), α10 (ITGA10), αE (ITGAE), αV (ITGAV), β1 (ITGB1), β3 (ITGB3) and β5 (ITGB5) in K562 cell line was performed by qRT-PCR. Briefly, total RNA was extracted using Trizol reagent based on the manufacturer's instruction and 2 µg of total RNA was reverse transcribed to synthesize cDNA by using 2 µL of mix-1 containing 0.5 µL random hexamer primer, 1 µL (10mM) dNTP's, 0.5 µL Oligo (dT) which was heated at 65 °C for 5 min. Then, 8 µL of mix-2 containing 4 µL synthesis buffer (5x), 2 µL DTT (0.1M), 1 µL RNase out and 1 µL M-MLV RT enzyme were added and incubated at 25 °C for 10 min, 37 °C for 50 min and 70 °C for 15 min. RT-PCR was carried out on a StepOnePlus (Applied Biosystems) RT-PCR system with human β-actin as endogenous housekeeping gene using the primers which were screened with the help of IDT (Integrated DNA Technologies) PrimerQuest Tool and NCBI Primer-BLAST are shown below:

Gene	Forward	Reverse
ITGA2B	CACGCATGGTTCAACGTGTC	CTGTGTCCACACCTGAGCTT
ITGA3	GCGCAAGGAGTGGGACTTAT	CTGCATCGTGTACCCAATATAGA
ITGA5	TGCCGAGTTCACCAAGACTG	TGCAATCTGCTCCTGAGTGG
ITGA10	TGGGTGTACCTAGGCAGTAT	ACTCTCTCTCCTGTCTGCTT
ITGAE	GCCTCCCTCTCCAAACATTCA	CTGAAACATGAGCAGATGACCTC
ITGAV	GTTGGGAGATTAGACAGAGGAAAG	GCAGACGACTTCAGAGAATAGG
ITGB1	GCCTGTTAGACATGACTGATGA	TTTCCCATGGCCTTTGTAGAT
ITGB3	CATCACCATCCACGACCGAA	GGTTGTTGGCTGTGTCCCAT
ITGB5	GATGACACCACAGGAGATTGC	GGTAGTACAGGTCCACAGGA
β-actin	GCGAGAAGATGACCCAGAT	CCAGTGGTACGGCCAGA
GAPDH	TCACTGTTCTCTCCCTCCGC	TACGACCAAATCCGTTGACTCC

10 µL of reaction mixture containing 5 µL master mix SYBR Green, 2 µL of 10 × 10^{-6} m primers, and 3 µL of 5 ng µL^{-1} cDNA template was added in triplicates to the MicroAmp Fast Optical 96-well reaction plate. The reaction mixtures were heated at 95 °C for 10 min before

proceeding through 40 cycles of the denaturation step, 95 °C for 15 s, and annealing/elongation step, 60 °C for 1 min.

The integrin-β1 mRNA levels following polymer/siRNA treatment in K562 and MDA-MB-231 cells were analyzed using the described RT-PCR protocol at desired time points (days 1, 3 and 6). Briefly, K562 cells (incubated just prior to siRNA treatment) and MDA-MB-231 cells (seeded 24 hours prior to siRNA treatment) were treated with the siRNA complexes for a pre-determined time, and cells were harvested for RT-PCR analysis. $\Delta C_{T,}$ $\Delta\Delta C_T$ and Relative Quantity (RQ) of mRNA were calculated with respect to the housekeeping gene, while the no treatment (NT) group served as the reference group.

2.6. siRNA uptake

The siRNA delivery efficiency was studied using FAM-labeled control (scrambled) siRNA in both cell lines. MDA-MB-231cells were allowed to attach and grow for 24 hours, after which polymer/FAM-siRNA complexes prepared at a weight/weight ratio of 9:1 were added to the cells at 60 nM, with unlabeled control siRNA serving as a negative control. K562 cells were collected and centrifuged at 1400 rpm for 5 min whereas MDA-MB-231 cells were centrifuged following trypsinization and collected in complete medium. Both the cell lines were washed with HBSS and fixed with 3.7% formaldehyde. The uptake was quantified using BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA). The uptake of FAM-siRNA was determined and expressed as mean siRNA levels per cell (in arbitrary fluorescent units) and as percentage of cell population positive for FAM-labeled siRNA with cells carrying unlabeled siRNA complexes designated as 1% positive population.

2.7. Cell surface integrin-β1 analysis by flow cytometry

K562 cells were added to the wells along with polymer/siRNA complexes whereas MDA-MB-231 cells were seeded 24 hours prior to the treatments. Both cell lines were treated with the polymer/siRNA complexes for 3 days, following which K562 cells were collected by centrifugation and MDA-MB-231 cells were collected following trypsinization. Three days of silencing was optimal to detect the differences in cell surface integrin-β1 in MDA-MB-231 cells in our previous study, so that immunostaining was performed following 3 days of treatment in K562 cells in this study [28]. The cells were washed with HBSS, followed by 1 hour of staining with PE-labeled Integrin-β1 antibody at room temperature according to the manufacturer's protocol. Cells were fixed with 2% formaldehyde prior to analysis with BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA). The extent of integrin-β1 expression was calculated as a percentage of mean fluorescence intensity relative to no treatment samples.

2.8. hBMSC binding assay

The K562 cells were treated in 48 well plates with the desired polymer/siRNA complexes and harvested after 3 days of treatment. hBMSC were seeded in 96-well flat bottom plates and were maintained for 2-3 days to reach confluency [29]. The treated K562 cells were counted using the trypan blue exclusion assay and equal number of cells were stained with DiI dye (carbocyanine dye) for 20 min, after which they were washed twice with the HBSS. These cells were added to the confluent hBMSC monolayers in 96-well plates and incubated for 1 hour at 37 °C. The plates were then placed upside down and further incubated for 3 hours at 37 °C. The non-adherent cells were removed by washing and the fluorescence was recorded at excitation of 536 nm and emission of 607 nm using Fluoroskan Ascent plate reader (Thermolab systems).

2.9. K562 detachment assay

hBMSCs from different donors were seeded on a 6-well plate and was grown for 24 hours till they reached 60% confluency, following which K562-GFP cells were seeded on this monolayer and allowed to attach for additional 24 hours. The unattached/floating K562-GFP cells were removed and fresh complete media was added followed by complex treatment for 3 days. Unattached/floating cells were once again removed, followed by the addition of fresh media and further incubated for 2 days. Finally, K562-GFP cells were removed from the hBMSC monolayer and washed with HBSS to attain complete collection of the cells and the GFP fluorescence was recorded at excitation of 485 nm and emission of 510 nm using Fluoroskan Ascent plate reader (Thermolab systems). Percentage of detachment was calculated relative to non-treatment group. The cells that remained attached to the monolayer was collected by trypsinization and analyzed for GFP fluorescence by BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA).

2.10. Cell viability analyzed by MTT and trypan blue exclusion assay

To develop a K562 cell line resistant to Dasatinib (DA) treatment due to hBMSC binding, we cultured K562-GFP cells on a monolayer of hBMSC for 1, 3 and 5 days and the unattached cells were removed followed by DA addition at 5 nM and 10 nM. DA treatment which was allowed for 4 days under each treatment group separately. Dead cells percentage was calculated by staining the cells with 0.0002% trypan blue (final concentration) and analyzed by flow cytometry. Cell population positive to GFP as well as trypan blue dye was calculated for this purpose.

K562-WT cells were treated with complexes having 60nM of siRNA for 2 days followed by Imatinib addition at 300 nM, 500 nM and 1000 nM. Cells were further incubated for an additional 3 days after which cell viability was accessed by MTT assay. Briefly, 1mg/ml final concentration of MTT was added to the cells and incubated at 37 °C for 2 hours. Cells were collected by centrifugation at 1400 rpm for 5 minutes, supernatant was removed and the pellet containing the crystals was dissolved in DMSO by vortexing and absorbance was recorded at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments).

2.11. Methylcellulose colony-formation unit (CFU) assay

The K562 cells were allowed to attach and grow on fibronectin-coated plates for 2 days, following which they were treated with polymer/siRNA complexes for 3 days. Cells were collected by HBSS washing, counted using trypan blue dye exclusion assay and equal number of viable cells were seeded with 400 µL of Methylcellulose and allowed to form colonies at 37 °C. Colony counts were assessed using a light microscope on day 7 and day 14.

2.12. Statistical analysis

All results are summarized as mean ± standard deviation and unpaired Student's *t*-test was used to assess the statistical differences between the group means with p-value < 0.05 considered as statistically significant. Where specified, the number of independent experiments used to generate the data (n) are indicated. Pearson's correlation coefficient was calculated where indicated.

3. Results

3.1. K562 cells can effectively bind to fibronectin at relatively short duration

To assess the fibronectin binding ability of K562 cells, the cells were seeded on fibronectin coated wells and bound cells were quantified at different time points (4h, 8h, 20h and 24h). The cells retained in wells without fibronectin coating (WO-FN; Figure 2A) was minimal which remained at similar levels for all the time points. The corresponding microscopic images confirmed this result (Figure 2B). In contrast, the

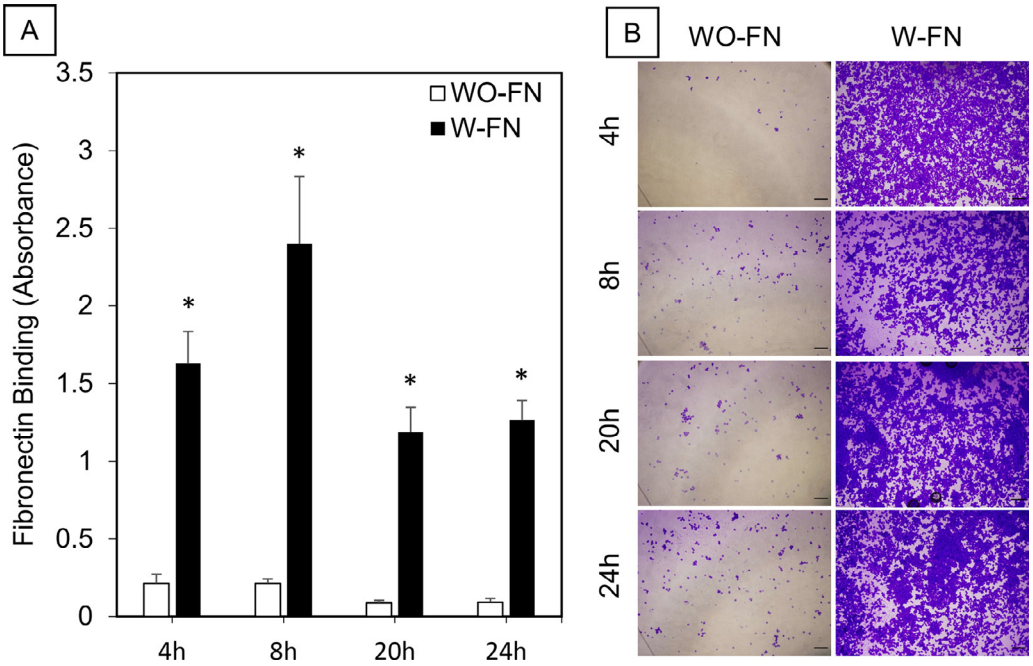


Fig. 2. Fibronectin binding ability of K562 cells at different time points. (A) The number of cells bound to fibronectin as given by relative absorbance units and (B) the corresponding microscopic images. The results are summarized for without fibronectin (WO-FN: white bars) and with fibronectin (W-FN: black bars) coated wells. $^{*}p \leq 0.05$, N=2. Scale bar: 20μm.

wells with fibronectin coating (W-FN; Figure 2A) showed large number of cells binding to fibronectin which was observed as early as 4h. The attachment was not also altered with increase in time. The corresponding microscopic images (Figure 2B) provided a confirmation on the fibronectin binding ability of the K562 cells, which suggest the presence of cell surface receptors (presumably integrins) that could help in this binding.

3.2. Integrin-β1 (ITGB1) is highly expressed in K562 cells

Following the confirmation of fibronectin binding, we investigated the presence of potential integrins that could play a role in the fibronectin binding. Hence, we assessed the levels of mRNA transcripts of various integrins in K562 cells. The RQ of mRNA levels were calculated with respect to β-actin housekeeping gene. Integrin-β1 was highly expressed when compared to other integrin genes, followed by integrin-α5 and integrin-α_V (Figure S1). Other integrins analyzed such as ITGA2B, ITGA3, ITGA10, ITGAE, ITGB3 and ITGB5 had very low or no expression (Figure S1). Given the well-established role of integrin-β1 in fibronectin binding [30–34], this analysis suggests that integrin-β1 could be primarily responsible for the fibronectin binding ability of the K562 cells. A similar level of Integrin-β1 was also observed when K562 cells were cultured in contact with hBMSC (Figure S1, insert)

3.3. Successful delivery of siRNA into K562 Cells

The high expression of fibronectin-binding integrin-β1 makes it a viable target for siRNA-mediated knockdown, which would help to reduce the ECM binding ability of K562 cells. As the siRNA delivery is dependent on the efficiency of gene delivery vehicle, we used several lipid-modified PEIs in this study to identify optimal delivery system. The 1.2PEIs that had linoleic acid, α-linoleic acid, propionic acid substitutions and their respective redox-sensitive disulfide cross-linked (-ss-) polymers were used for siRNA delivery. MDA-MB-231 cells were used as reference cell phenotype due to the high expression of integrin-β1 on their cell surface as shown in our previous studies [28]. The widely used commercial reagent Lipofectamine, and the in-house prepared polymer 1.2PEI-αLA4 which exhibited high siRNA uptake in MDA-MB-231 cells as well as in K562 cells from previous studies [35,36] were used as reference carriers. Among the investigated polymers, 1.2PEI-tLA6, 1.2PEI-tαLA6, 1.2PEI-tαLA6-ss exhibited the highest siRNA uptake as shown by the mean fluorescence intensity in K562 cells (Figure 3A-i). The same polymers showed high siRNA uptake in MDA-MB-231 cells as well, in addition to 1.2PEI-tLA2 and 1.2PEI-tαLA2 (Figure 3A-ii). The correlation in the siRNA delivery between the K562 and MDA-MB-231 was calculated ($r^2 = 0.7118$) and found to be significant ($p < 0.0001$). The three polymers 1.2PEI-tαLA6-ss, 1.2PEI-tLA6 and 1.2PEI-tαLA6 (indicated by A, B and C in Figure 3B) worked best in both cell types. The size of these lipid-modified PEI/siRNA complexes ranged between 300 to 350 nm with a positive zeta-potential, which is favorable for efficient siRNA uptake in both cell types [37,38]. The complexes from the lipid-modified polymers also remained stable *in vivo,* based on our previous study for breast cancer treatment [39].

3.4. Significant knockdown of integrin-β1

The knockdown of integrin-β1 was performed with 1.2PEI-tαLA6 and redox-sensitive -SS- cross-linked derivatives as they may yield better siRNA release inside the cell, resulting in better knockdown [24]. Scrambled siRNA (CsiRNA) was used as control in this experiment. Following 3 days of treatment with polymer/siRNA complex, cells were stained with PE-integrin-β1 antibody and analyzed by flow cytometry. We assessed the cell surface integrin-β1 levels in K562 and MDA-MB-231 cells (Figure 4A) and observed a drastic difference. K562 cells had readily detectable expression of integrin-β1, but this amount was lower than the levels found in MDA-MB-231 cells. In K562 cells, the polymers 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss showed significant knockdown of the cell surface integrin-β1 (Figure 4B-i and Figure S2). These polymers also had high siRNA uptake in K562 cells (see Figure 3A-i) and they also exhibited strong silencing in MDA-MB-231 cells (Figure 4B-ii). The observed knockdown was achieved with a single treatment of polymer/siRNA complex having 60 nM siRNA concentration, as a single siRNA treatment was found to be sufficient to perform integrin-β1 knockdown and further increase of siRNA concentration did not affect

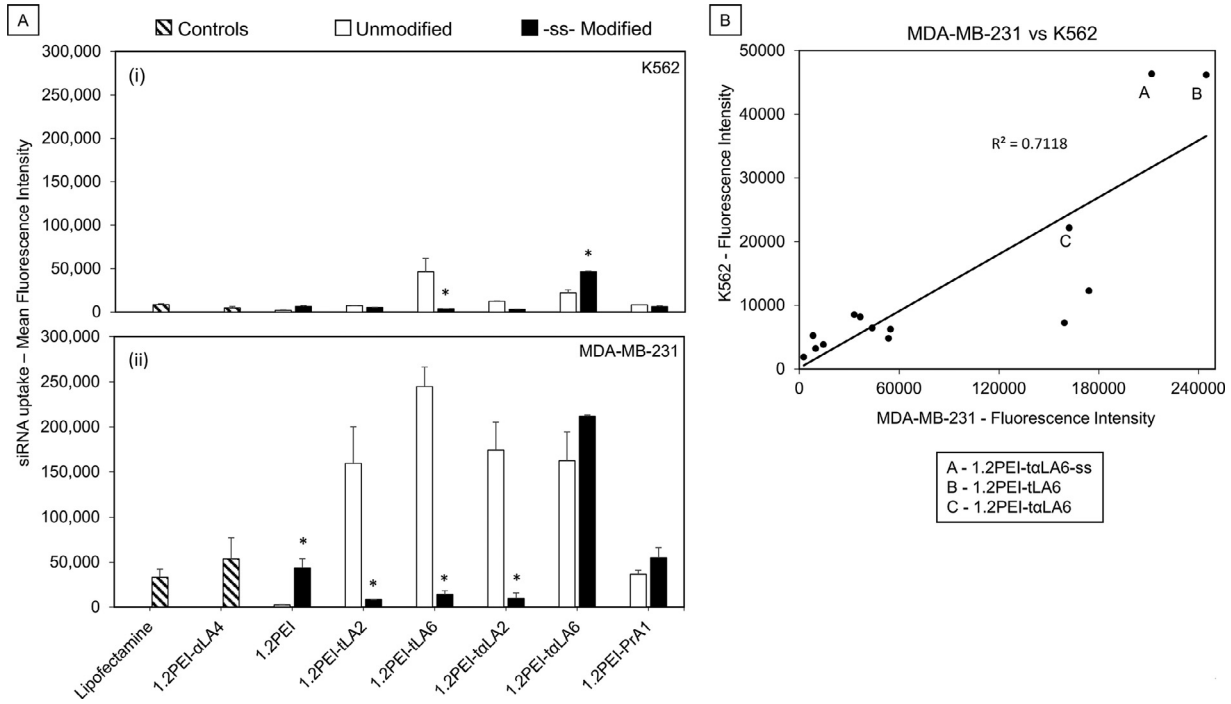


Fig. 3. The uptake of FAM-labelled siRNA after 24 hours of treatment. (A) Mean fluorescence intensity of siRNA in K562 (i) and MDA-MB-231 (ii) cell lines. Control polymers (striped bars), non-disulfide crosslinked polymers (white bars) and -ss- modified polymers (black bars) $^{*}p \leq 0.05$ and (B) The correlation of mean fluorescence between the two cell lines was calculated by Pearson's correlation coefficient ($R^2 = 0.7118$ with $p < 0.0001$).

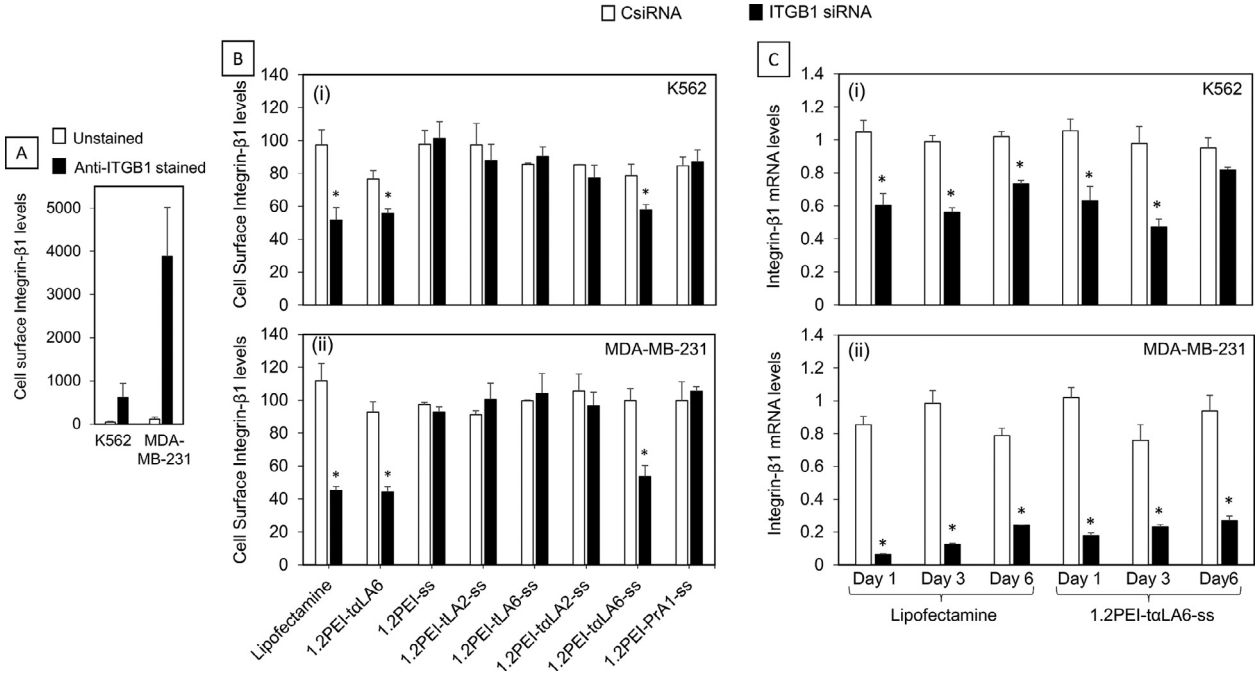


Fig. 4. (A) Cell surface integrin-β1 levels in K562 and MDA-MB-231 cells. Cells without antibody staining are denoted by white bars, and cells stained with PE-integrin-β1 antibody are denoted by black bars. (B) Integrin-β1 silencing with siRNA delivery, as evaluated by cell surface immunostaining and flow cytometry. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment group) were measured after 3 days of siRNA treatment in (B-i) K562 and (B-ii) MDA-MB-231 cells. (C) The mRNA levels of integrin-β1 after 3 days of siRNA treatment with the 1.2PEI-tαLA6-ss was quantified through qRT-PCR (values are plotted relative to no treatment group) in (C-i) K562 and (C-ii) MDA-MB-231 cells. Control siRNA (scrambled; CsiRNA) is shown as white bars while integrin-β1 siRNA in black bars. A polymer/siRNA (weight/weight) ratio of 9:1 with 60 nM of siRNA concentration was used. $^{*}p \leq 0.05$, N=2.

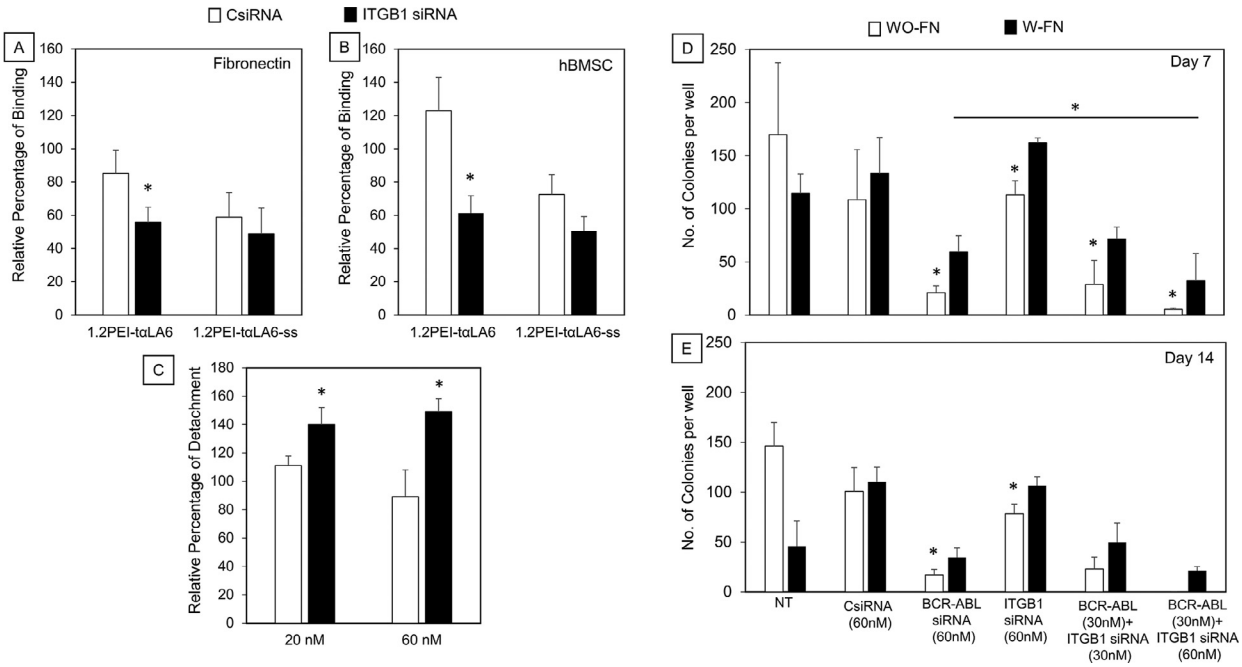


Fig. 5. Effect of integrin-β1 silencing on the binding ability of K562 cells. (A) Binding on fibronectin-coated surfaces: Percentage of K562 cells binding to fibronectin after 3 days of treatment with siRNA using the polymers 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss, N=4. (B) Binding towards hBMSCs: Percentage of K562 cells binding to human bone marrow stromal cells (hBMSCs) after 3 days of siRNA treatment. (C) Detachment of K562-GFP cells from hBMSC monolayer following 3 days of complex treatment with two different siRNA concentrations and 5 days of attachment. Values are plotted as percentage of cells detached relative to no-treatment group (taken as 100%), $^{*}p \leq 0.05$, N=3. (D & E) Combinational siRNA treatment and its effect on the number of colony forming K562 cells. Cells cultured without fibronectin (WO-FN) are represented by white bars and with fibronectin (W-FN) are represented by black bars. Colony counts at (D) Day 7 and (E) Day 14. CsiRNA: Control scrambled siRNA.$^{*}p \leq 0.05$, N=2.

the outcome at the cell surface levels (Figure S3). Furthermore, we explored the application of optimal (1.2PEI-tαLA6-ss) polymers for other targets, for which we delivered survivin siRNA and observed comparable knockdown of survivin to that of the control transfection agent (Figure S4), which highlights the high efficacy of lipid-modified polymers irrespective of the target.

We additionally evaluated the effect of polymer/siRNA complex on integrin-β1 mRNA levels at different time points, starting from day 1 to day 6 after a single treatment. In K562 cells (Figure 4C-i), the mRNA levels can be reduced significantly at day 1 and day 3, but the silencing effect was not significant at day 6 for 1.2PEI-tαLA6-ss, but the knockdown was still present for the Lipofectamine. The knockdown efficiency was higher (at the same dose of siRNA) and more prolonged from day 1 to day 6 in MDA-MB-231 cells (Figure 4C-ii), which was also observed in our previous study.

3.5. Functional outcome of integrin-β1 knockdown in K562 cells

Following the successful knockdown of both cell surface protein and mRNA levels of integrin-β1, the functional outcome of this effect was studied by assessing K562 binding to fibronectin and hBMSC. K562 was shown to bind to hBMSC, which contributes towards the unresponsiveness to drug treatment in the late stage CML and could eventually lead to disease relapse [40–42]. The integrin-β1 reduction helped to significantly decrease its binding ability towards fibronectin and hBMSC when treated with 1.2PEI-tαLA6 polymer (Figure 5A, B). Similarly, the silencing of integrin-β1 was able to detach the K562 cells from hBMSC monolayer (Figure 5C).

After assessing the detachment of cells from hBMSC monolayer, we also analyzed the cells that remained attached to the monolayers as an indirect measure of detachment. The samples having high number of detached cells from the monolayer should exhibit low number of the cells in the well, as most of the cells have been removed due to ITGB1 silencing. Four sources of BMSCs were used in this study. In hBMSC-1, ITGB1 silencing helped to detach most of the K562 cells at 60 nM siRNA treatment. However, with hBMSC-2, no reduction was observed using 60 nM of siRNA concentration (Figure S5-A). Using different sources of hBMSC and with different concentrations of siRNA (20, 40, 60 and 80 nM), we could observe that hBMSC-3 responded to ITGB1 silencing by decreasing the percentage of attached cells, unlike the K562 cells on hBMSC-4 (Figure S5-B).

The therapeutic outcome of silencing integrin-β1 in combination with BCR-ABL was studied by colony formation assay. K562 cells that were cultured without fibronectin (WO-FN; white bars) showed a significant drop in the number of colonies when treated with BCR-ABL siRNA/polymer complexes (60 nM siRNA) (Figure 5D, E). A drop in the colony forming ability of K562 cells on fibronectin (W-FN; black bars) was also seen when the cells were treated with BCR-ABL siRNA, but the decrease in this case was less compared to cells grown in the absence of fibronectin (at both day 7 and day 14). Treatment with integrin-β1 siRNA alone didn't reduce the colony counts at both time points. A combination of BCR-ABL (30 nM) and integrin-β1 (30 nM) siRNA/polymer complex resulted in colony counts similar to BCR-ABL siRNA/polymer complex treatment alone. Whereas an increase in the integrin-β1 siRNA concentration to 60 nM along with BCR-ABL siRNA of 30 nM resulted in significant reduction of colony counts than the BCR-ABL siRNA/polymer complex treatment alone at day 7 (Figure 5D). Therefore, reduced responsiveness to BCR-ABL siRNA treatment observed due to fibronectin binding can be overcome by a combinational knockdown of integrin-β1 in K562 cells (Figure 5D, E).

4. Discussion

The development of drug resistance in CML was considered to be primarily due to point mutations on the BCR-ABL domain, which alters the binding ability of TKIs. Recent studies have also shown BM microenvi-

ronment to play an important role in binding of CML cells and contributing to the observed drug resistance [19,40]. The binding of K562 cells with fibronectin, which is a key molecule that regulates the adhesion process in BM niche via cell surface integrin-β1, conferred resistance to IM treatment in the *in vitro* studies [30,43]. In our initial study to confirm the fibronectin binding ability of CML cells, we could observe a sharp increase in the number of cells binding to fibronectin within a very short period of 4 hours. The sustained binding to fibronectin (from 4 to 24h) could also signify that these cells can stay attached for longer periods of time. Earlier evidence have also shown the fibronectin binding ability of *BCR-ABL* positive cells, providing protection against IM-induced cell death and a decrease in the proliferation which could be a possible survival mechanism to evade drug treatment [13,30]. The binding of CML cells with the aid of integrins could activate various intracellular signaling pathways altering the expression of several genes such as P-gp, Mcl-1, Bcl-xl, and survivin, all of which are involved in IM resistance in CML cells [19]. In addition, the combined *BCR-ABL*/fibronectin signaling can induce morphological changes (cytoskeletal function) in leukemic cells leading to integrin clustering and increased cell adhesion which could also contribute to enhanced resistance to TKI treatment [17,44]. Hence, binding to fibronectin may lead to multiple changes in the CML cells, which necessitates the identification of key integrins involved in this process. Thus, PCR analysis to identify the potential integrin(s) responsible for fibronectin binding were conducted and the results revealed high expression of integrin-β1 in K562 CML cells. Compelling evidences of integrin-β1 as a key molecule that aids in the binding of CML cells to BM/fibronectin, transform them into a viable target to overcome drug resistance. The fact that integrin-β1 mediated pathway was shown to mediate fibronectin binding by various studies [31–34,36], regulate degradation of Bim pro-apoptotic protein levels [30], prevent apoptosis in *BCR-ABL* positive cells [13], all contributed to our focussing on integrin-β1 as a therapeutic target.

Although integrin-β1 was identified as a key receptor for BM attachment of CML cells, the role of other components of BM microenvironment in drug resistance was also explored by others. The cytokines produced in BM was shown to aid in the survival of *BCR-ABL* positive leukemic cells even in the presence of TKIs and this resistance was reversed, when leukemic cells were isolated from BM and exposed to IM [45]. In addition, culturing of K562 cells in bone marrow stroma-derived conditioned medium resulted in upregulation of STAT3 and increased levels of its target genes Bcl-xl, Mcl-1 and survivin which eventually leads to IM resistance [46]. Hence, the signals received from the BM initiates various downstream signaling pathways which provides *BCR-ABL* independent survival, conferring resistance to TKIs. Targeting of downstream signaling pathways such as PI3K-Akt-mTOR pathway which is shown to be activated in CML cells after IM treatment helps in the survival and resistance, and targeting this pathway resulted in growth inhibition of CML cells [47,48]. Other intracellular pathways such as ERK1/2 and SMAD have also shown to sustain resistance to IM induced apoptosis when CML cells were cultured with the bone marrow stroma cells [40]. All these studies involved direct growth and interaction of CML cells with BM microenvironment or use of bone marrow stroma-conditioned medium, which the CML cells in contact with BM, in turn activates various signaling pathways for drug resistance. Thus, we speculate that the disruption of the interaction/binding of CML to BM will have therapeutic outcome for overcoming drug resistance.

The other significant integrins expressed in K562 were integrin-α5 and integrin-α_V (Figure S1), which suggest the possible heterodimerization with integrin-β1 on cell surfaces. An earlier study on cell adhesion-mediated drug resistance in CML unveiled that the binding of K562 cells to fibronectin was via integrin-$\alpha_5\beta_1$ and this contributed to the resistance towards DNA damaging agents such as melphalan, miozantrone and γ-irradiation [41,49]. The knockdown of the single, highly expressed integrin-β1 will reduce its availability to heterodimerize with the other α-subunits. One might additionally have to target other integrins such as integrin-α_5 for increased potency to reduce fibronectin/hBMSC adhesion and this will be explored in our future studies.

For the successful delivery of siRNA into K562 cells, we used lipid-modified 1.2 kDa PEI polymers as they exhibit inherent buffering capacity that helps in endosomal release of the nucleic acid via “proton-sponge” effect [50]. Multiple lipid-modified PEIs and corresponding disulfide (-ss- modified) linked counterparts were screened for this purpose. Lipid substitution on low molecular weight PEIs enhances their cellular binding and permeability across the plasma membrane, whereas the disulfide cross-linked polymers can provide better release of nucleic acid inside the cell due to redox sensitivity of the disulfide linkages [24,51]. In this study, we could observe a significant increase in siRNA uptake after disulfide crosslinking of 1.2PEI-tαLA6 polymer, whereas opposite effect was observed with 1.2PEI-tLA6 polymer (decreased siRNA uptake) (Figure 3A-i). In attachment-dependent breast cancer cells MDA-MB-231, disulfide crosslinking had positive effect only with 1.2PEI, whereas detrimental effects were observed with 1.2PEI-tLA2, tLA6 and tαLA2 polymers (Figure 3A-ii). With 1.2PEI-tαLA6 and PrA1, the siRNA uptake was unaltered after disulfide crosslinking (Figure 3A-ii). Previous studies with disulfide cross-linked polymers showed comparatively similar results, were the transfection efficiency of 2 kDa PEI was improved post disulfide cross-link, whereas palmitoyl chloride modified PEI did not improve its transfection efficiency [24]. Similar observation were made with disulfide cross-linked 800 Da PEI; the transfection efficiency was improved with disulfide cross-linking and cytotoxicity lowered [51,52]. Based on the comparative siRNA delivery in MDA-MB-231 and K562 cells, select polymers emerged with high siRNA delivery in both cell lines, while other lipid-modified polymers did not exhibit high siRNA delivery either in K562 cells or MDA-MB-231 cells. Clearly, the efficiency of the delivery agents appeared to be highly dependent on the nature of lipid substituent.

The knockdown of integrin-β1 was performed with the disulfide crosslinked polymers, as they were shown to improve the transfection efficiency and exhibit better intracellular release of the nucleic acid in other studies [51,53]. In K562 cells, the significant knockdown observed with the disulfide cross-linked 1.2PEI-tαLA6-ss polymer was comparable to its counterpart 1.2PEI-tαLA6 (Figure 4B-i). Similar results were observed with MDA-MB-231 cells which associates with the siRNA uptake of these 2 polymers. In both cell types, the knockdown efficiency was comparable to that of the commercial transfection reagent Lipofectamine RNAiMAX/2000. As the aim of this study was to evaluate the therapeutic potential of integrin-β1 to overcome drug resistance in CML, we did not explore the various physicochemical properties of the polymers used in this study. To evaluate the knock-down of integrin-β1, reduction in integrin-β1 mRNA levels (Figure 4C-i) correlated with cell surface knockdown (Figure 4B-i) as well as with high siRNA uptake (Figure 3A-i) in K562 cells. A similar outcome was observed with the MDA-MB-231 albeit at much higher extent of silencing (Figure 4C-ii). This difference could be explained by the high levels of integrin-β1 in MDA-MB-231 when compared to the moderate/low levels in K562 cells (Figure 4A) as well as ~5-fold increased siRNA delivery in the breast cancer cells with equivalent delivery systems.

The main objective of this study was to examine the possible reduction of K562 cell binding to hBMSC by integrin-β1 knockdown and improvement of sensitivity to treatment. A single treatment with 60 nM of siRNA targeting integrin-β1 was able to reduce the hBMSC binding of K562 CML cells (Figure 5B) as well as the detachment of hBMSC-attached K562 cells (Figure 5C). Significant reduction was observed only with 1.2PEI-tαLA6 polymer treatment and not by 1.2PEI-tαLA6-ss polymer which could be explained by the reduced binding observed even with control siRNA (CsiRNA) treated cells, indicating a non-specific effect caused by disulfide cross-linked polymer. Furthermore, the detachment of K562 cells from hBMSC was confirmed in half of the patient samples by the reduced amount of K562 cells that remained attached to hBMSC following integrin-β1 knockdown (Figure S5-A, B).

We cultured the K562 CML cells on fibronectin, followed by treatment with polymer/siRNA complexes in order to perform colony formation cell assays. This is a widely used method to study the proliferation and differentiation of hematopoietic cells by looking into the colony counts, which is depended on the ability of K562 cells to form colonies when seeded in a semisolid medium (methylcellulose) [54]. As expected, the knockdown of *BCR-ABL* drastically reduced the colony counts in the absence of fibronectin. However, the presence of fibronectin substrate for cell attachment provided resistance to BCR-ABL siRNA treatment which was observed at day 7 as well as day 14 (Figure 5D, E). Treatment with integrin-β1 siRNA alone, did not affect the colony formation of K562 cells but the presence of fibronectin increased the colony counts which is due to the favorable growth conditions provided by fibronectin [13,16,30,55]. The significant decrease in the colony counts while using a combinational treatment of BCR-ABL (30 nM) with integrin-β1 siRNA (60 nM) highlighted the therapeutic potential of integrin-β1 silencing. But this effect was not observed at day 14, when the colony counts for some groups started to decline. The drastic decline in the colony counts at day 14 of no treatment (NT) when cultured on fibronectin is an interesting observation which could be due to proliferation inhibition due to its binding to fibronectin as reported earlier [56]. Due to the ability of leukemic cells to evade TKIs by residing in the bone marrow niche, we aimed to induce TKI resistance by growing K562 cells on hBMSC for different time points and exposing them to the DA (5 and 10 nM). Furthermore, we also examined the effect on cell viability with a combinational treatment of IM at 3 different concentration and integrin-β1 silencing. However, we were unable to show that binding to hBMSC would make the K562 cells less effective to TKI treatment in our hands and no difference in cell viability between IM alone, CsiRNA and integrin-β1 treatment was observed (Figure S6).

In conclusion, we showed that fibronectin was capable of enhancing the binding ability of K562 CML cells in this study. Since this interaction is generally mediated by the presence of integrin-β1, its successful knockdown with the aid of polymeric siRNA delivery helped to decrease its binding to fibronectin, as well as hBMSC, and the detachment of leukemic cells from the stromal layer. The combinational silencing of BCR-ABL and integrin-β1 was found to reduce colony formation especially in the presence of fibronectin. All these outcomes should be further tested in patient cells as well as animal studies to validate the therapeutic potential of integrin-β1 and the polymeric systems to address drug resistance in CML. If the reported results could be successfully translated to these models, targeting integrin-β1 in addition to *BCR-ABL* oncogene could be a viable treatment for drug resistant CML clones.

Declaration of Competing Interest

HU and RBKC are founders of a start-up company (RJH Biosciences) commercializing the polymers for nucleic acid delivery. Other authors declare that there are no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbiosy.2021.100021.

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