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Delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting bcr–abl fusion gene in Chronic Myeloid Leukemia cells

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ABSTRACT

Gene silencing by RNA interference (RNAi) is a promising therapeutic approach for a wide variety of diseases for which the biological cause is known. The main challenge remains the ineffective RNAi delivery inside the cells. Non-viral gene delivery vectors have low immunogenicity compared to viral vectors, but are constrained by their reduced transfection efficiency. Silencing of the bcr–abl gene expression by RNAi confers therapeutic potential in Chronic Myeloid Leukemia (CML), but is limited by the cytotoxicity of the existing delivery methods. Here, we present evidence that the fusion between the cell penetrating peptide (CPP) HIV-Tat (49–57) and the membrane lytic peptide (LK15), Tat–LK15, mediates high transfection efficiency in delivering short hairpin RNA (shRNA) and small interfering RNA (siRNA) targeting the BCR–ABL oncoprotein in K562 CML cells. Our results show that shRNA complexes induce a more stable gene silencing of bcr-abl when compared to silencing mediated by siRNA complexes. In addition, silencing of the BCR-ABL oncoprotein by both shRNA and siRNA delivered by Tat-LK15 is more efficient and longer lasting than that achieved using Lipofectamine and more importantly without considerable cytotoxicity. In these terms Tat–LK15 can be an alternative to DNA/siRNA delivery in difficult-to-transfect leukemic cells.

1. Introduction

Chronic Myeloid Leukemia (CML) is a hematopoietic malignant disease characterized by the presence of the Philadelphia chromosome (Ph) where the reciprocal translocation of chromosomes 9 and 22 results in the formation of the BCR–ABL fusion oncoprotein, which exhibits deregulated tyrosine kinase activity [1,2]. The BCR–ABL fusion protein has been attributed to causing the pathogenicity of this disease and creates the challenge to target BCR–ABL protein therapeutically. Current treatment for CML includes tyrosine kinase inhibitor Imatinib Mesylate (Gleevec) [3] and a range of second generation inhibitors like Dasatinib and Nilotinib [4]. Nevertheless growing resistance to this treatment calls for the development of improved therapy [1].

Thus, an alternative is the use of RNA interference (RNAi) as a gene therapy model. RNAi is the process of suppressing the expression of a protein by using double stranded RNA (dsRNA). The elucidation of the function of several genes using RNAi has triggered considerable interest in the scientific community to utilize RNAi for therapeutic applications as the increasing number of clinical trials using RNAi indicates [5]. Indeed, it appears to be a valuable tool to tackle the growing problems in cancer related diseases as it can inhibit the production of over-expressed genes [6]. The RNAi pathway can be achieved using small interfering RNA (siRNA) [7] or short hairpin RNA (shRNA) [8] both of which are believed to result in different efficiencies and different silencing timescales.

This aspect is crucial for K562 CML cells, which are known to be difficult to transfect [9]. Indeed K562 have been conventionally transfected by harsh methods such as electroporation [10], lentiviral vectors [11] and lipid-based transfection methods [2,12] for the delivery of shRNA or siRNA. However, these methods of gene delivery have their own limitations. Electroporation is not practical for use *in vivo* [13] and viral vectors pose the risk of the viral gene being integrated into the host genome [14]. Consequently, non-viral gene delivery is a reasonable alternative to viral vectors due to their lower cytotoxicity [13], but the obstacle in this approach is the low transfection efficiency of the non-viral vectors. Delivery of different cargoes to various cell types has been carried out using cell penetrating peptides (CPPs) which stretch up to 30 amino acids long and have the ability to translocate cell membranes [15]. The cationic nature of CPPs allows them to electrostatically bind to the phosphate backbone of the nucleic acids to form a stable complex in which nucleic acids are protected from degradation [16]: CPPs have been shown to effectively deliver DNA [17] and siRNA *in vitro* [18] and *in vivo* [9]. Additionally, covalent attachment of CPPs to other cationic peptides increases their transfection efficiency and prevents DNA neutralization by its negative charge [19]. Recent work in our laboratory combined covalent attachment of CPP Tat-derived peptide (amino acids 49–57 of the HIV-1 TAT protein (trans-activating

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transcriptional activator protein of human immunodeficiency virus type-1) [20]), to cationic membrane active peptide LK15 [21]. The transfection efficiency of the Tat–LK15 peptide generated by this fusion was increased by two orders of magnitude compared to the Tat peptide alone in human colorectal adenocarcinoma, HT29, and human connective tissue fibrosarcoma, HT1080, cell lines [22].

Here we assessed the efficacy of the Tat–LK15 peptide in delivering siRNA and shRNA expression plasmid targeting the BCR–ABL fusion oncoprotein in K562 cells and studied the extent of its cytotoxicity. Furthermore, we compared the Tat–LK15 transfection efficiency to that of Lipofectamine, a lipid-based transfection reagent. Although there are no standard transfection reagents for siRNA delivery, Lipofectamine was chosen based on its documented high ability to mediate DNA transfection, which could also be utilized to study siRNA delivery. Tat–LK15/shRNA plasmid complex silenced the bcr–abl gene expression up to 90% after 96 h of transfection and lasted for 192 h as shown at the protein level by western blot analysis. On the other hand, up to 70% reduction of the BCR–ABL protein levels was observed using siRNA-based complexes 48 h after transfection using varying amounts of siRNA.

Our results suggest that stable silencing of the BCR–ABL oncoprotein is achieved by Tat–LK15/shRNA plasmid compared to Lipofectamine based complexes. This conclusion is also valid for short term silencing with siRNA-based complexes. Tat–LK15, with further work *in vivo*, could be a potential gene therapy tool.

2. Materials and methods

2.1. Cell culture

Human chronic myelogenous leukemia cell line K562 (ATCC) was grown in RPMI 1640 (Invitrogen, UK) supplemented with 10% v/v Foetal Bovine Serum (FBS), 1% v/v Penicillin/Streptomycin and 2 mM L-Glutamine (Invitrogen, UK) at 37 °C in a humidified air atmosphere containing 5% CO_2.

2.2. shRNA cloning

The shRNA sequence targeting the B3A2 isoform of the BCR–ABL fusion protein was designed based on previously published information [23].

Targeting Sequence: B3A2: 5′ GCAGAGTTCAAAAGCCCTT.

The shRNA insert comprises the loop sequence of 5′ CTCAAGAGA 3′ and was flagged by BamHI and HindIII restriction sites at the 5′ and 3′ ends respectively. The final sequences were then annealed and cloned into the pSilencer 2.0 U6 hygro (Applied Biosystems, UK). A shRNA insert which has no homology to any known sequences in human, mouse or rat was used to construct a negative control shRNA plasmid. This sequence was cloned into the pSilencer 2.0 U6 hygro vector as mentioned above. This plasmid was used as a negative control for all shRNA transfection experiments.

2.3. siRNA

The siRNA was synthesized by Dharmacon (UK) that matched the B3A2 target region as covered by the shRNA. The siRNA sequence used was: 5′ GCAGAGUUCAAAAGCCCUU 3′. Negative control siRNA was purchased from Sigma (All stars negative control, Sigma, UK).

2.4. Peptide

CPP HIV-Tat (49–57) (RKKRRQRRR) was fused to membrane active peptide LK15 (KLLKLLLKLLLKLLK) using the linker GGG between the two sequences. Tat–LK15 peptide (RKKRRQRRRGGGKLLKLLLKLLLKLLK-$CONH_2$) having a net positive charge of +13 was synthesized by Advanced Biomedical (UK) and was >95% pure.

2.5. Vector/oligonucleotide complex preparation

The method in the present work has been described elsewhere [22]. Briefly, to achieve a 1:1 charge ratio, 0.81 μg of Tat–LK15 peptide was dissolved in 50 μL of HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4). 1 μg of oligonucleotide (DNA or siRNA) was resuspended using 10 mM HEPES buffer pH 7.4 to a final volume of 50 μL. The peptide solution was then added to the oligonucleotide solution, agitated for a few seconds and left to incubate at room temperature for 30 min. This solution was then added to 900 μL of Opti-MEM (Invitrogen, UK) containing cells.

For higher oligonucleotide charge ratios, increasing amounts of oligonucleotide were diluted in 50 μL of 10 mM HEPES buffer pH 7.4 and subsequently higher amount of peptide depending on the amount of oligonucleotide and the charge ratio.

Lipofectamine™ and oligonucleotide complexes were prepared according to the manufacturer's instruction (Invitrogen). For a 1:1 weight ratio, 1 μg of Lipofectamine™ was diluted in 50 μL of Opti-MEM-1, mixed with 1 μg of DNA or siRNA diluted in Opti-MEM and incubated for 15 min at room temperature.

2.6. YOPRO-1 displacement assay

Oligonucleotides were labelled using YOPRO-1 at a ratio of 50:1 dye/base pairs for DNA and a ratio of 5:1 for siRNA. Increasing amounts of vector were added as described in the Vector/Oligonucleotide Complexes Formation section and the fluorescence intensity was measured using the count rate provided by Fluorescence Correlation spectroscopy (Zeiss Confocor 2).

2.7. Transfection

K562 cells were plated at a cell density of 1.5×10^6 cells in 900 μL of reduced serum Opti-MEM® I in a 6 well plate (Corning, UK). 100 μL of peptide/DNA complexes were added to the cells and incubated in a 37 °C incubator for 4 h. After 4 h, the medium was removed and the cells washed with PBS and resuspended in 2 mL of RPMI supplemented with 10% FBS without any antibiotics and incubated for 96 h and 192 h for shRNA based transfection and 48 h for siRNA-based transfection replacing with fresh complete medium every 72 h.

2.8. Size and zeta potential measurement

Solutions of peptide/DNA complexes were prepared as described above at a DNA concentration of 10 μg/mL in 10 mM HEPES buffer pH 7.4. Particle size was determined by dynamic light scattering, and zeta-potentials were measured by electrophoretic method using the Zetasizer 3000 HS (Malvern Instruments, UK). Results are expressed as the average of three measurements.

2.9. Cy5 nucleic acid labelling

pcDNA3 plasmid was labelled using the Cy5 nucleic acid labelling Kit (LabelIT, Mirus Bio, USA) according to manufacturer's instructions with slight modifications. In particular, a 10:1 dye to DNA base pair ratio was used and the resulting complexes were incubated at 37 °C for 2 h. 100% recovery of labelled DNA was assumed as per manufacturer's instructions. B3A2 siRNA was labelled using the same procedure but at a 1:2 ratio. Transfection was performed using the Cy5 labelled DNA and siRNA as mentioned above.

2.10. Flow cytometry analysis

Cells were plated at a density of 1.5×10^6 cells in 900 μL of Opti-MEM in a 6 well plate. The desired vector/labelled nucleic acid complex was then added to the cells and incubated for 4 h. After 4 h,

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the cells were rinsed twice with PBS and then cells were trypsinized using 0.1% v/v trypsin/EDTA in PBS for 5 min at 37 °C to remove any membrane-bound complexes and then washed twice with PBS. RPMI supplemented with 10% v/v foetal calf serum (FCS) was then added to each well and the cells were centrifuged at 1000 rpm for 5 min. After discarding the supernatant the cell pellet was resuspended in ice-cold PBS (0.5 ml). Fluorescence analysis was performed immediately at a minimum of 10,000 events/sample using fluorescence-activated cell sorter (FACS) (Becton Dickinson, UK) set up to detect red fluorescence (Ex 633 nm, Em 670 nm). Cell viability was determined based on live cells being gated based on forward and sideward scatter.

2.11. Western blotting

Cells were harvested using high salt lysis buffer (50 mM Tris HCl pH 7.4, 240 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 supplemented with freshly added 1 mM DTT, 1 mM Phenylmethanesulfonyl fluoride, and approximately 1 μg of protease inhibitor per cell lysate). Cells were then lysed for 25 min at 4 °C and then cell debris removed by centrifugation at 13,000 rpm at 4 °C for 15 min. Protein concentration was estimated using Bradford Assay Reagent and absorbance was measured at 595 nm. Protein sample was mixed with half volume of 3× sample buffer (187 mM Tris pH 6.95, 30% glycerol, 15% β mercaptoethanol and 6 mg/mL of SDS and 0.01% bromophenol blue) and denatured by boiling at 95 °C for 5 min. Equal amounts of proteins were loaded on a 7.5% w/v polyacrylamide gel alongside a PageRuler™ Plus prestained protein ladder (Fermentas, UK). Samples were transferred onto a Immobilon-P transfer membrane (Millipore, UK), block using 5% dried skimmed milk and probed using anti-BCR–ABL primary antibody (Santa Cruz Biotechnology Inc, USA) (1:1000) and anti-α-Tubulin primary antibody (Abcam, UK) (1:10,000) and incubated at 4 °C overnight. Membranes were washed 5 times for 5 min each at 4 °C in 2.5% dried skimmed milk PBS-Tween (1× PBS 0.1% Tween 20) and stained with Anti Rabbit secondary antibody conjugated to horseradish peroxidase (GE Life Sciences, UK) (1:10,000). The membrane was again washed 5 times for 5 min each using PBS-Tween and treated with West Pico chemiluminescent substrate (Thermo Fisher Scientific, UK) and visualized on a Fujifilm Super RX medical film.

2.12. Quantitative RT-PCR

K562 cells were transfected using vector and shRNA plasmid and incubated in Opti-MEM for 4 h. Cells were washed with PBS and incubated with RPMI supplemented with 10% FBS. Cells were lysed after 48 h for shRNA plasmid transfected cells. Cytoplasmic RNA was extracted using the RNeasy Mini kit (Qiagen, UK) using the protocol provided by the manufacturer.

RNA concentrations were measured and 1 mg/mL of RNA was reverse transcribed according to the two-step protocol (ABgene) using an oligo-dT primer (ABgene). The DNA was diluted 4-fold and used for qPCR analysis using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma). B3A2 was measured using the following primers: forward primer for B3A2: 5′ CAT CGT CCA CTC AGC CAC T 3′ and reverse primer: 5′ ACG AGC GGC TTC ACT CAG 3′. The following primers were used to measure Rpl19: forward primer for Rpl19: 5′ ATG TAT CAC AGC CTG TAC CTG 3′ and reverse primer: 5′ TTC TTG GTC TCT TCC TCC TTG 3′. The signal obtained from B3A2 primer was normalised using the values obtained from Rpl19 primers.

2.13. Image analysis

Intensities of the bands obtained from the Western Blot were analyzed using the Image J software (NIH, USA). First, the background of the image was reduced. Bands in a single vertical lane were selected using the rectangular selection tool and box of the same dimensions was used for the remaining lanes. Then, the lanes were plotted and the area of each peak was determined after eliminating the background intensity. The intensity of BCR–ABL bands was then divided by intensity of the housekeeping gene α-Tubulin.

2.14. Cytotoxicity studies

Vector and vector/nucleotide cytotoxicities were determined using the colorimetric (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) (Sigma Aldrich, UK) assay as described by van de Loosdrecht et al. [24]. Briefly K562 cells were transfected as described above and incubated with the desired treatment for 4 h at 37 °C. The assay was performed after 24 h post-transfection for the vector/nucleic acid complex while for the peptide cytotoxicity the assay was performed 4 h post-transfection. Then MTT was added at a final concentration of 0.5 mg/mL and incubated for 4 h or until a dark purple precipitate can be visualized. The supernatant was removed without disturbing the purple formazan crystals and 150 μL of DMSO was added. The plate was kept on a shaker at 150 rpm for 20 min at room temperature. The absorbance was measured using a plate reader at 550 nm. The percentage of cell viability of test sample was calculated by using the formula: $A_{test} / A_{control} \times 100$.

2.15. Statistical analysis

Statistical differences between the mean values of different groups were evaluated by unpaired Student's *t*-test. A p-value ≤ 0.05 was considered significant.

3. Results

3.1. Vectors/DNA complexes formation

Non-covalent complex formation was studied by competitive binding assay where the vector displaces YOPRO-1, an intercalating fluorescent dye on the labelled nucleic acid [25]. Fig. 1 shows that upon addition of increasing amount (charge ratio) of polycation, the fluorescence intensity decreased thus indicating that competition between Tat–LK15 or Lipofectamine™ and YOPRO-1 takes place. Complete displacement/quenching was achieved at lower charge ratios for DNA based complexes than for siRNA-based complexes indicating quicker complex formation for DNA and vector. Based on Fig. 1, 2:1 and 3:1 charge ratios were used for DNA and siRNA respectively for subsequent studies.

3.2. Size of the complex and zeta potential

The size (dynamic light scattering) and charge (zeta potential) of the vector/DNA and vector/siRNA complexes at the above charge

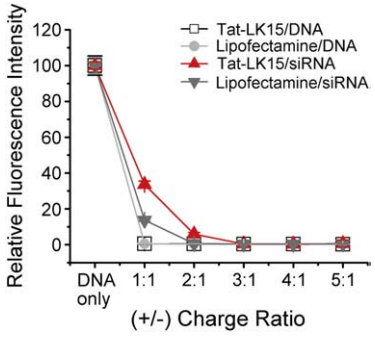

Fig. 1. YOPRO-1 displacement assay. DNA and siRNA were labelled using YOPRO-1 (one molecule of dye for every 50 base pairs for DNA and one molecule of dye for every 5 base pairs for siRNA.) Increasing amounts of Tat–LK15 and Lipofectamine™ were added according to the increase in charge and weight ratios respectively and fluorescence intensity was measured. The values represent the mean ± SD., $n = 10$.

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ratios were characterized. Tat–LK15/DNA complexes at a (+/−) charge ratio of 2:1 and Tat–LK15/siRNA at a (+/−) charge ratio of 3:1 were used to find the size of the complexes as shown in Table 1. The size of complexes of Tat–LK15/DNA and Tat–LK15/siRNA formed at a (+/−) charge ratio of 2:1 and 3:1, respectively, did not show any significant difference ($p>0.05$). However the difference in zeta potential exhibited by the Tat–LK15/siRNA complex at 3:1 and the Tat–LK15/DNA complex at 2:1 was very significant ($p<0.05$) indicating that the complex formed at 3:1 has a high net positive charge which could influence its cellular uptake.

3.3. DNA uptake

The ability of Tat–LK15/DNA and Lipofectamine™/DNA complexes to be taken up by K562 cells was examined using Fluorescence Activated Cell sorter (FACS) 4 h post-transfection. DNA dose dependence was tested as Cy5 labelled DNA was used at increasing amounts (1 μg, 2 μg and 5 μg) for each vector at the optimal charge ratios (2:1, see above). Fig. 2 shows the profiles, the percentage of cells that had taken up complexes and the mean fluorescence intensity of these cells (indication of the amount of Cy5 labelled DNA taken up by cells).

Overall FACS profiles (Fig. 2a and b) indicated different pattern of uptake for Tat–LK15 based and Lipofectamine™ based complexes. Indeed, the histograms for Tat–LK15 based complexes are broad while for Lipofectamine™ based complexes, histograms have a more classic "bell shape" centred at lower fluorescence intensity. In Fig. 2c, a clear DNA dose dependence is observed for Tat–LK15 based complexes as the mean fluorescence intensity (MFI) increases with the DNA amount. When incubated with the Lipofectamine™/DNA complexes the dose dependence of the MFI is not clear initially but obvious between 2 and 5 μg of DNA. It is worth noting that except for 2 μg DNA, the MFI is higher when using lipofectamine than when using Tat-LK15 as transfection agent. On the other hand, the percentage of positive cells (Fig. 2d) is always higher after incubation with Tat–LK15/DNA complexes than with Lipofectamine™ based complexes but appears not to change drastically with increasing amounts of DNA used, reaching a maximum of 80% cell uptake using 5 μg of labelled DNA for Tat–LK15 based complexes. Consequently, Tat–LK15 enables the delivery of DNA to larger population of cells than Lipofectamine™.

Similarly, the uptake of Cy5-labelled siRNA mediated by Tat–LK15 and Lipofectamine™ was studied at a charge and weight ratio of 3:1 respectively using flow cytometry (Fig. 3). Fig. 3a and b shows similar patterns of uptake for Tat–LK15- and Lipofectamine™-based complexes (except small peaks can be observed for Lipofectamine™ based complexes at high intensities). As observed in Fig. 2c, the MFI (Fig. 3c) indicated that a clear siRNA dose dependence for cells incubated with Tat–LK15/siRNA complexes as fluorescence intensity increases with amounts of siRNA. The percentage of positive cells also showed a concentration dependent increase for Tat–LK15/siRNA transfected cells reaching a maximum of about 60% of the cell population (Fig. 3d). At high siRNA concentrations, both Lipofectamine™- and Tat–LK15-based complexes showed similar levels of uptake and percentage of positive cells.

Despite the fact the siRNA or shRNA labelled with Cy5 (a pH-independent dye) may demonstrate narrower distributions [22] and although protocols provided by the manufacturer to remove any unbound Cy5 from the solution were followed (protocol from Mirus, US), the FACS profiles (Figs. 2a and 3a) showed a very large distribution of the fluorescence intensity which can be explained by considering both the vector and the cells. Firstly, the vector influences the distribution in two ways: i) complexes resulting in electrostatic interactions between the cationic vector and the negatively charged nucleic acids have a broad size distribution (heterogeneity) as also observed by Combez et al. [18] (see average and standard deviation in Table 1). Additionally, the level of fluorescence of each complex is likely to be affected by quenching of which, the extent will depend on the size of the complex. Thus the combination of these two aspects (size and quenching) is likely to result in a broad distribution of fluorescence levels. Secondly, the cell cycle phase or the cell populations may affect the fluorescence profiles thus leading to broad distributions.

3.4. Gene silencing

3.4.1. Silencing using the shRNA expression vector

shRNA expression plasmids have the advantage of continuous synthesis of dsRNA leading to RNAi pathway and thus a more stable silencing can be expected [26]. However, they are limited by the nuclear delivery required for the expression of the plasmid [26,27]. CPPs have shown to deliver plasmid DNA into the nucleus and resulted in gene expression in various cell lines [17]. Here we use Tat–LK15 which has shown high transfection efficiencies when compared to CPP Tat and membrane lytic peptide LK15 [22].

Equal amount of DNA was transfected to all cells (30 μg) and the amount of shRNA (encoding) plasmid varied from 0 to 30 μg for each transfection. The vector/DNA complexes were formed at a charge (+/−) ratio of 2:1. Cells were harvested 96 h post-transfection and western blot analysis was performed to detect the protein levels of p210 BCR–ABL. Increasing the amount of shRNA plasmid from 10 μg to 30 μg resulted in a reduction of BCR–ABL protein levels from 75% to 90% while no effect was observed with the negative control shRNA (Fig. 4a). qRT-PCR performed on mRNA extracted 48 h post-transfection has also shown a reduction in levels of B3A2 target mRNA from 70% to 85% compared to the mock treated cells (Fig. 5).

On the other hand, in the presence of Lipofectamine™, the positive control vector, protein levels were reduced by about 25% and 75% when treated with 10 μg and 20 μg of shRNA respectively 96 h post-transfection while again no effect of the negative control shRNA was observed (Fig. 4b). K562 cells treated with Lipofectamine/siRNA showed a reduction in levels of B3A2 mRNA ranging from 60% to 90% with increasing amounts of shRNA treated.

3.4.2. Eight days of gene silencing using Tat–LK15 and shRNA plasmid complex

One of the main advantages of using shRNA expression plasmid is the continuous expression of the shRNA and hence a more efficient silencing of the expression of the target gene. To confirm that more efficient silencing for longer time was conferred by shRNA transfection we followed the BCR–ABL oncoprotein levels in cells transfected with Tat–LK15/shRNA at 2:1 (+/−) charge plasmid complex ratio, 192 h post-transfection. Efficient gene silencing of 85% was observed using Tat–LK15/shRNA complexes 192 h post-transfection only with the highest amount of specific RNA sequence (Fig. 4c). On the other hand, bcr–abl gene silencing using Lipofectamine™/shRNA plasmid complex reached a maximum of 40% using 10 μg of shRNA plasmid whereas transfection with higher amounts of shRNA expression plasmid led to an increase in BCR–ABL protein levels (Fig. 4d).

Table 1
Average diameter (nm) and zeta potential (mV) of DNA complexes prepared in 10 mM HEPES buffer-saline (pH 7.4) using a (+/−) charge ratio of 2:1 at a DNA concentration of 10 μg/ml. Tat–LK15/siRNA complexes prepared at a ratio of 3:1 at a final siRNA concentration of 10 μg/ml in 10 mM HEPES-buffered saline (pH 7.4). The values represent the mean ± SD., $n=3$.

Vector	(+/−) charge ratio	Size (nm)	Zeta potential (mV)
Tat–LK15/DNA	2:1	850 ± 141.4	+4.09 ± 0.834
Tat–LK15/siRNA	3:1	663.4 ± 256.1	+10.55 ± 2.61

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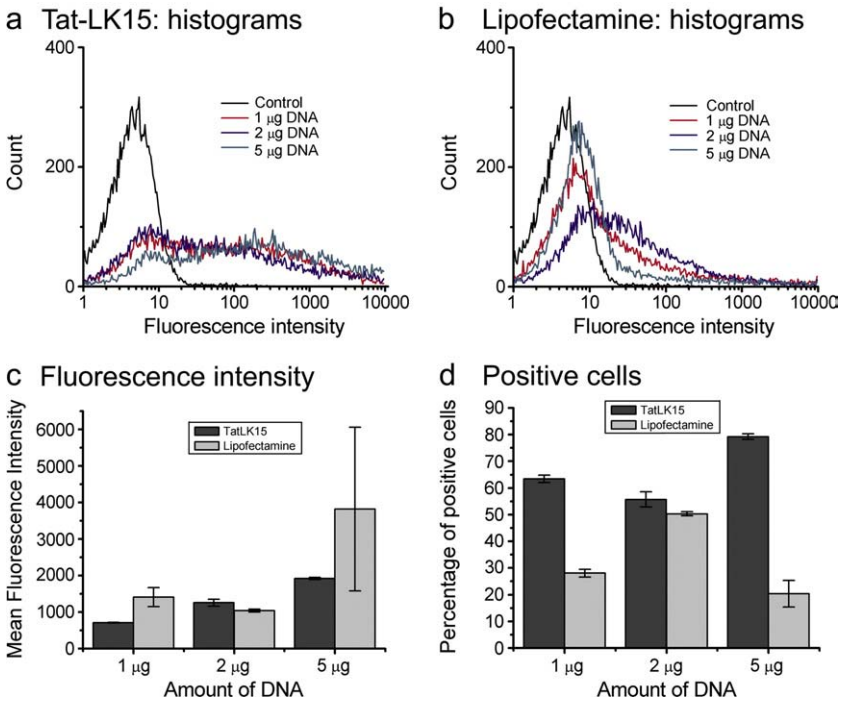

Fig. 2. a and b**:** Representative histograms obtained from the flow cytometry to study the uptake of DNA in K562 cells using Tat–LK15 (a) and Lipofectamine™ (b). pcDNA3 was labelled using Cy5 nucleic acid labelling kit at a ratio of 0.1:1 as described in Materials and methods section. Vector/DNA complexes were formed as described in Materials and methods section. Flow cytometry was performed 4 h after transfection. c: Graph showing the mean fluorescence intensity of labelled DNA uptake by Tat–LK15 and Lipofectamine™ with increasing amounts of DNA. d: Graph showing the percentage of positive cells having the Cy5 labelled DNA. The values represent the mean ± SD., $n = 3$.

3.4.3. Transient gene silencing using siRNA

To balance the stable gene silencing induced by shRNA, it appeared necessary to compare the transfection efficiency and gene silencing of both the shRNA plasmid and siRNA-based complexes. Fig. 1 indicates that complete displacement of YOPRO-1 can be observed at (+/−) charge ratios higher than 2:1. Transfection at this charge ratio resulted in only 50% bcr–abl gene silencing (data not shown) 48 h-post transfection. However, at a higher (+/−) charge ratio of 3:1,

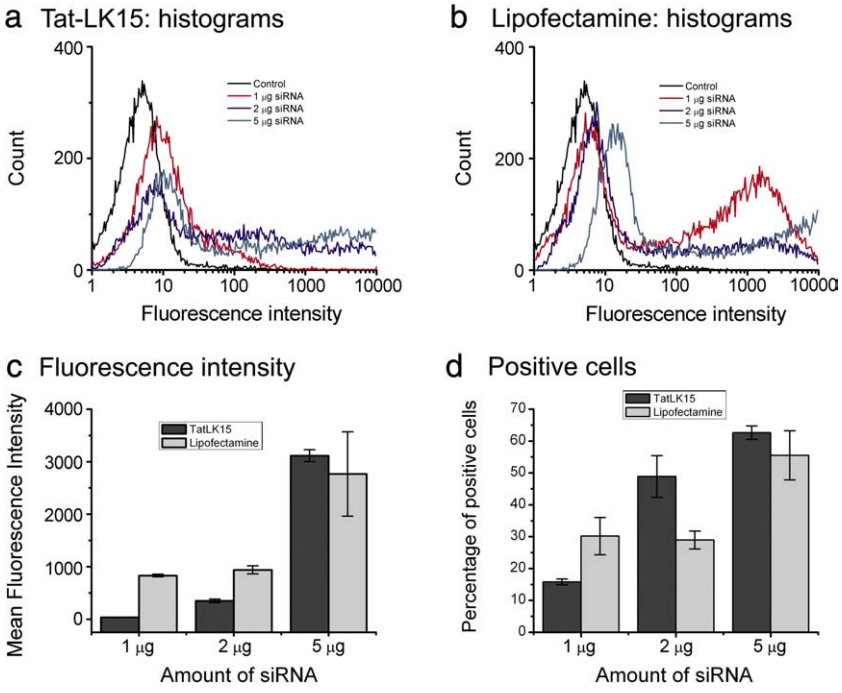

Fig. 3. a and b**:** Representative histograms obtained from the flow cytometry to study the siRNA uptake in K562 cells using Tat–LK15 (a) and Lipofectamine™ (b). B3A2 siRNA was labelled using Cy5 nucleic acid labelling kit at a ratio of 0.5:1 as described in Materials and methods section. Vector/siRNA complexes were formed as described in Materials and methods section. Flow cytometry was performed 4 h after transfection. c: Graph showing the mean fluorescence intensity of labelled siRNA uptake by Tat–LK15 and Lipofectamine™ with increasing amounts of siRNA. d: Graph showing the percentage of positive cells having the Cy5 labelled siRNA. The values represent the mean ± SD., $n = 3$.

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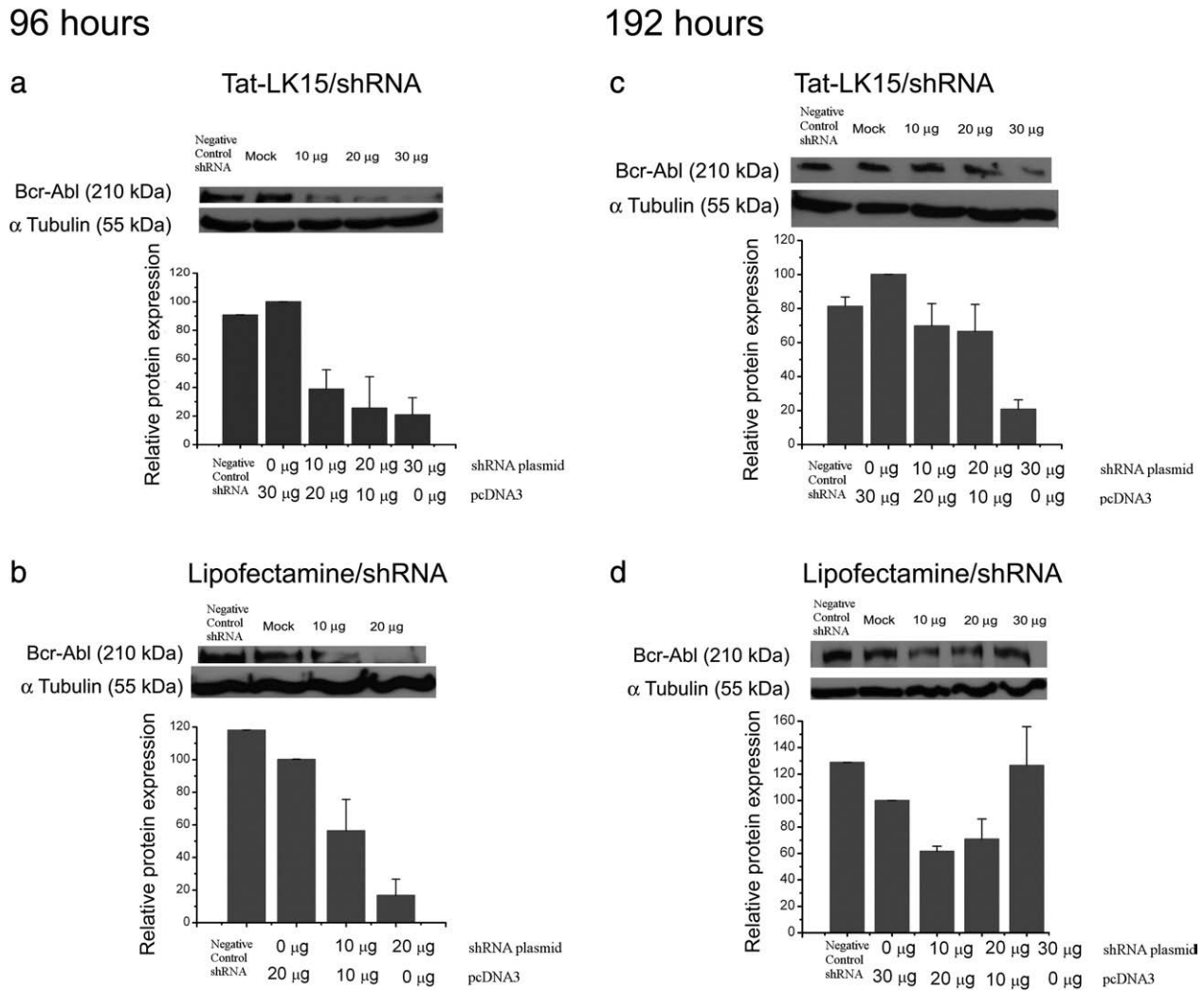

Fig. 4. a and b: Western blot showing the expression of p210 BCR–ABL (molecular weight 210 kDa) and control protein α-Tubulin (molecular weight 56 kDa). Extract from cells transfected using Tat–LK15/shRNA expression plasmid (a) at a (+/−) charge ratio of 2:1 and Lipofectamine™/shRNA plasmid (b) at a weight ratio of 2:1 respectively. Cells are harvested and checked for gene silencing 96 h post-transfection using western blot. c: Western blot of cells transfected using Tat–LK15/shRNA expression plasmid at a (+/−) charge ratio of 2:1 and harvested at 192 h post-transfection. The left column is obtained using 30 μg of negative control shRNA plasmid. d: Cells transfected using Lipofectamine™/shRNA expression plasmid at a weight ratio of 2:1 and harvested 192 h post-transfection. The densitometry readings obtained from the western blot using Image J software indicated by bar charts show that the reduction in protein levels normalised to the expression levels of α-Tubulin. All transfections were compared to 30 μg of negative control shRNA plasmid. Graph shows data obtained from three independent experiments. Western blots shown are a representative result.

transfection using Tat–LK15/siRNA has a significant silencing of at least 70% for amounts varying from 1 μg to 30 μg of siRNA at 48 h post-transfection with a maximum reduction between 5 and 10 μg as shown in Fig. 6. It is worth noting that gene silencing could not be observed after 48 h. Subsequent transfection studies on K562 using siRNA led to significant decrease in cell viability making detection of protein levels on western blot difficult. The same pattern of gene silencing was observed using Lipofectamine™/siRNA complexes at 48 h post-transfection.

3.5. Cytotoxicity studies

To determine whether cytotoxicity had any effect on our results, MTT assays were performed 24 h post-transfection with vector/DNA and vector/siRNA complexes.

Fig. 7a shows that there are no significant cytotoxicities observed for complexes prepared with shRNA plasmid with both vectors. However, the difference in cytotoxicities by shRNA and siRNA complexes is significant ($p<0.005$) showing that the siRNA-based complexes are more toxic than the shRNA based complexes. However, it is difficult to compare cytotoxicities due to the higher charge ratio used for siRNA-based complexes (3:1) when compared to the shRNA based complexes (2:1), because higher net positive charge on the complex could have an effect on the cytotoxicity.

Additionally, an investigation of Tat-LK15 peptide toxicity (including concentrations equivalent to those used for transfection complexes) showed that the cytotoxicity is high at concentrations of Tat–LK15 peptide above 10 μM alone as seen in Fig. 8. This high toxicity in using only the peptide could be due to its highly cationic nature and membrane lytic activity of Tat–LK15 at high concentrations.

4. Discussion

Chronic Myeloid Leukemia with the chromosomal translocation and the presence of bcr–abl fusion gene provides a suitable system to study gene therapy using different gene delivery vehicles. Recent developments using RNAi to target the BCR–ABL fusion protein have utilized electroporation [10] and viral vectors [10,28]. However, cytotoxicity and host gene integration [14] have limited their safety for *in vivo* models. Conversely, non-viral vectors such as CPPs offer low

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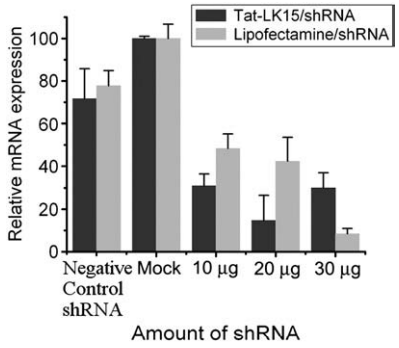

Fig. 5. qRT-PCR was performed using mRNA extracted from cells treated with vector/shRNA 48 h post-transfection. Dark grey bars indicate K562 cells transfected using Tat–LK15/shRNA and light grey bars indicate cells treated with Lipofectamine/shRNA complexes. Signal obtained using B3A2 primer was normalised with Rpl19 primer signal. Cells treated with negative control siRNA (30 μg) were used as control.

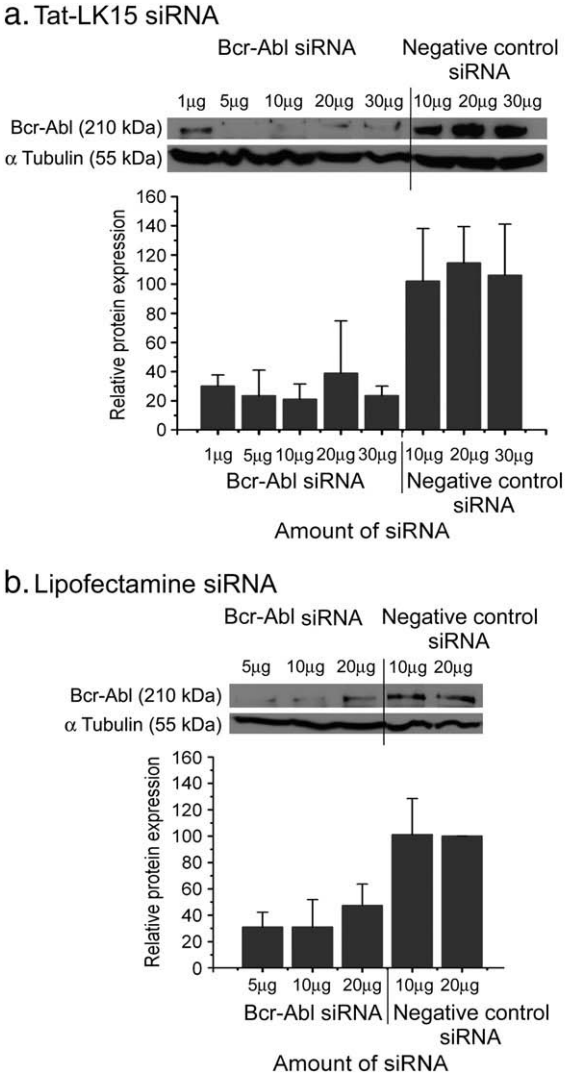

Fig. 6. a: Western blot showing the expression of p210 BCR–ABL (molecular weight 210 kDa), and control protein α-Tubulin (molecular weight 55 kDa) in K562 cells transfected with Tat–LK15/siRNA complex at a charge (+/−) ratio of 3:1. Cells were lysed 48 h post-transfection and analyzed using western blot as described in Materials and methods section. b: Cell extract from cells transfected with Lipofectamine™/siRNA complex at a weight ratio of 3:1 and harvested 48 h post-transfection. The BCR–ABL protein expression of B3A2 siRNA transfected cells was compared to cells treated using varying amounts of negative control siRNA as indicated on the right column. Graphs indicate data obtained from three individual experiments. Western blot shown here is a representative blot.

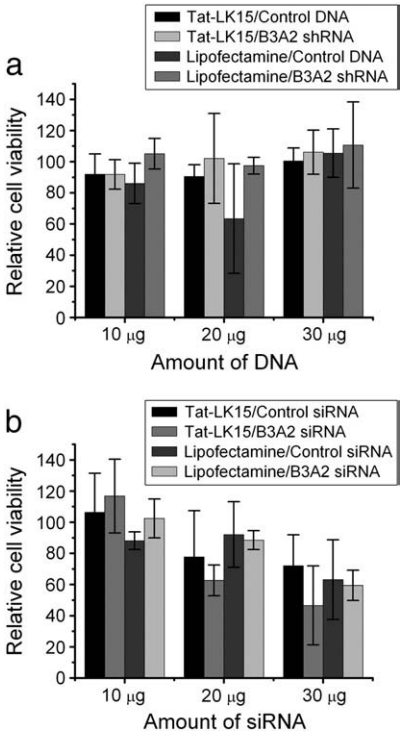

Fig. 7. a: Cytotoxicity of vector and DNA/siRNA complexes in K562 cells 24 h after transfection. MTT assay to study the cytotoxicity of Vector/DNA complexes using a (+/−) charge ratio of 2:1 for Tat–LK15 and weight ratio of 2:1 for Lipofectamine™ using increasing amounts of DNA observed 24 h after transfection. b: MTT assay to study the cytotoxicity of vector/siRNA complexes using a (+/−) charge ratio of 3:1 for Tat–LK15 and weight ratio of 3:1 for Lipofectamine™ observed 24 h after transfection. The values represent the mean ± SD., $n = 3$.

cytotoxicity and can be modified [29] to improve previously poor transfection efficiencies in a variety of cell lines [9,17]. Non-covalent complexing of DNA to CPPs can reduce their cell penetrating capability due to the masking of positive charges [19]. This can be overcome by complexing a cationic peptide to the CPP to increase the efficiency of delivery [19]. In a recent study from our lab, the fusion of the CPP HIV-Tat (49–57) to the cationic membrane active peptide LK15 (Tat–LK15) resulted in much higher transfection efficiency when compared to Tat and LK15 peptides alone [22]. These encouraging results triggered our interest to test the efficiency of Tat–LK15 to deliver shRNA plasmid and siRNA targeting a

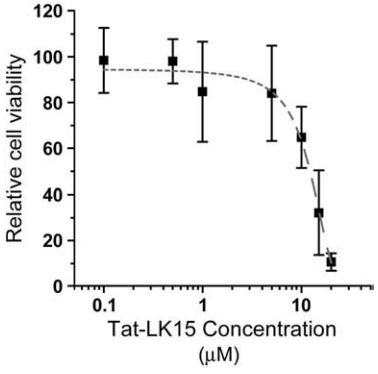

Fig. 8. Graph showing the relative (%) cell viability of vector in K562 cells tested 4 h post-transfection using the MTT cell viability assay. Cells transfected using varying concentrations of Tat–LK15 covering the concentration ranges used for the transfection experiments. Cells were incubated with varying concentrations of vector for 4 h at 37 °C. MTT was added to the cells and incubated for 4 h. The precipitate is then dissolved using DMSO and the absorbance measured at 550 nm. The values represent the mean ± SD., $n = 3$.

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therapeutically relevant protein in the difficult-to-transfect cell line K562 compared to Lipofectamine™.

Cytotoxicity study of Tat–LK15 alone shows that usage of the peptide at high concentrations can lead to cell toxicity (Fig. 8), which might be a result by the high density of positive charges (+13) on the peptide. This high concentration of peptide alone causing cell cytotoxicity has also been observed using HT29 cells using Tat–LK15 peptide, showing the similarity in the behaviour of the peptide across different cell lines used [22]. Indeed, these positive charges allow the formation of complexes with DNA and siRNA that facilitate the reduction of the toxicity associated with the large number of positive charges on the peptide (Fig. 7). However, positive charges are required to interact with the negatively charged proteoglycans on the cell membrane leading to cellular uptake [17]. This is achieved by using peptides at a charge ratio higher than electroneutrality for both DNA or siRNA-based complexes (Table 1). YOPRO-I competitive binding assay shows that Tat–LK15 displaces YOPRO-I effectively from DNA and siRNA from a (+/−) charge ratio of 1:1 thereby demonstrating the formation of intact complexes.

As observed from the flow cytometry studies, although Tat–LK15/DNA based complexes show lower total fluorescence intensity than Lipofectamine™, Tat–LK15 based complexes are taken up by a larger population of cells. This feature of Tat–LK15 is in agreement with the results reported from our lab on HT29 cells by Saleh et al. [22]. This higher uptake may explain the higher gene silencing efficiency using Tat–LK15/shRNA expression plasmid at 96 h compared to Lipofectamine™. Additionally, while studying the uptake of labelled siRNA mediated by both Tat–LK15 and Lipofectamine™, a similar trend in siRNA uptake was observed (Fig. 3). This can be correlated with the same level of siRNA-induced gene silencing by Tat–LK15 and Lipofectamine™ (Fig. 6). shRNA expression plasmid induced gene silencing was only detected 96 h post-transfection; this delay in silencing may be caused due to the long half-life of BCR–ABL protein [12] and also the post-transcriptional modifications by the shRNA to form a functional siRNA. Targeting this fusion protein by shRNA expression plasmid has lad to silencing of 90% at the protein level using 30 μg of shRNA expression plasmid and Tat–LK15 complex at 96 h (Fig. 4a). In contrast to Tat-LK15 based systems, Lipofectamine™/shRNA plasmid complexes lead to 75% silencing at 96 h post-transfection (Fig. 4b).

Interestingly, using the shRNA expression plasmid along with Tat–LK15 has led to 80% gene silencing even at 192 h post-transfection. Thus confirming that one of the main advantages of using a shRNA plasmid based expression system is continuous synthesis of mRNA and hence more shRNA entering the RNAi pathway, thereby causing a more stable gene silencing than siRNA [26,30].

Silencing by siRNA is dependent on its delivery to the cytoplasm. Peptide based gene delivery agents that show high nuclear localization of DNA perform poorly for the delivery of siRNA to the cytoplasm. Hence, the same peptide based system is unlikely to be used effectively for both the delivery of shRNA expression plasmid and also siRNA. Transfection of Tat–LK15/siRNA at a (+/−) charge ratio of 3:1 resulted in >70% silencing of BCR–ABL for most concentration ranges tested (60 nM to 1.8 μM). Although, Tat–LK15/siRNA complexes gave efficient silencing at wide concentration ranges, the relatively large variation in silencing results makes the task of determining the most effective dosage difficult and hence lies the limitation with using Tat–LK15 based siRNA complexes when compared with peptides proposed by Divita [18,31] and Dowdy [9]. The most effective silencing appears to be around the lower concentration ranges where there is minimal toxicity. There is however increasing toxicity at higher concentrations for which similar level of gene silencing is observed. Consequently toxicity levels should always be tested when using this peptide. Notwithstanding, gene silencing was not observed after 48 h which may comfort the transient nature of siRNA-induced RNAi. Interestingly, only the Tat–LK15/siRNA-based complexes caused RNAi induced cell death.

5. Conclusion

In this manuscript, we have successfully utilized Tat fusion peptide, Tat–LK15 to study gene silencing of bcr–abl in CML cell line K562 when compared to Lipofectamine™. Tat–LK15 showed high cell uptake and high efficiency of gene silencing in K562 cells. Cellular uptake reached a level of up to 80% of positive transfected cells using Tat–LK15/DNA and a level of up to 60% of positive transfected cells using siRNA-based complexes for both Tat–LK15 and Lipofectamine™. The property of Tat–LK15 to transfect a large population of cells could be due to the presence of membrane lytic peptide LK15 that destabilises the plasma membrane to increase transfection efficiency of cells. As reported recently [22], Tat–LK15 retains the membrane lytic activity of LK15 even after complexation with DNA thereby favouring its high cellular uptake. Gene silencing of bcr–abl by Tat–LK15/shRNA complex resulted in over 80% silencing of the oncoprotein at 96 h which lasts as long as 192 h. However, silencing of BCR–ABL using Lipofectamine™/shRNA complex resulted in 80% at 96 h but only less than 50% silencing at 192 h. This long lasting silencing induced by the Tat–LK15/shRNA complex could be attributed to the high percentage of cells transfected by the complex. Interestingly, the transfection using Tat–LK15 and Lipofectamine™ based siRNA complexes leads to similar level of silencing of BCR–ABL at 48 h which did not last for longer durations showing the transient nature of siRNA mediated gene silencing. Hence, in Tat-LK15 was equally efficient in transfecting K562 cells compared to conventional transfection methods such as electroporation, or viral and lipid based vectors. Our results show that Tat–LK15 could be a potential gene delivery model to study gene delivery as well as gene silencing. Further studies in *in vivo* systems could shed light on the usage of Tat–LK15 as a potential gene therapy tool.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2010.04.011.

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