Lipid nanoparticle mediated siRNA delivery for safe targeting of human CML in vivo

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Supplementary information

Supplemental Methods

Transfection of cell lines

$1x10^6$ cells were seeded per well of a 12-well plate right before transfection. LNP-siRNA formulations were directly added to the full serum containing medium for transfection at the indicated siRNA concentrations. Cells were transfected by nucleofection for siRNA screening and validation. One million cells were resuspended in 100µl of the kit V nucleofector solution (Amaxa Nucleofector Kit Lonza, Cologne, Germany), mixed with 200nM of siRNA and subjected to nucleofection using the Nucleofector device (Lonza, Germany), according to the manufacturer's protocol.

LNP-siRNA formulation

Our lipid mixture contained a proprietary lipid mix (SUB9KIT, Precision Nanosystems, Vancouver, Canada, with minor modifications) containing an ionizable amino lipid, a phosphatidylcholine (1, 2-distearoyl-sn-glycero-3-phosphocholine, DSPC), cholesterol and a coat lipid (polyethylene glycol-dimyristoyl glycerol, PEG-DMG) at a molar ratio of 50:10:38.5:1.5 [1]. Ionizable cationic LNPs have pKa values below 7 that allow siRNA encapsulation at low pH and a relatively neutral surface at physiological pH. LNPs also contained DiI labeled lipids to monitor LNP uptake in cells. DiI-C18 from Life Technologies (D-282) (0.2 mol% total lipid) was used to prepare fluorescently labelled LNPs, 0.2mol% 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-disulfonic acid (DiI-$C_{18)}$) from Invitrogen was directly added to the lipid mix. DiI is attached to the lipids of the LNPs. DiI has an excitation wavelength of 549 nm and emission wavelength of 565 nm. We used LNP formulations with 1.5% PEG-DMG for in vitro and in vivo use and to increase stability and circulation half-life time in vivo. LNPs were packaged with siRNA using a microfluidic system for controlled mixing conditions on the NanoAssemblr™ instrument (Precision Nanosystems, Vancouver, Canada). LNP-siRNA formulations were formed by injecting the lipid/ethanol solution into the first inlet, and a low pH aqueous buffer (sodium acetate buffer 25mM, pH 4) containing siRNA in the second inlet of the microfluidic chip (Figure 2A). The microfluidic

mixing took place at a flow rate of 12ml/min, with siRNA to lipid flow ratio of 3:1. The formed LNP-siRNA formulation was transferred to 10,000 MWCO dialysis cassettes (Slide-A-Lyzer, Thermo Scientific, IL, USA) and dialyzed against phosphate-buffered saline for 12–24 hours at 4ºC. The LNPs were sterile filtered using a 0.22micron syringe filter before use. Encapsulation efficiency was calculated by determining the free or non-encapsulated siRNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes, Eugene, OR) to the siRNA-LNP (Fi) and comparing this value to the total siRNA content that is obtained upon lysis of the LNP by 1% Triton X-100 (Ft). Percent encapsulation was calculated by the formula (Ft – Fi)/Ft x 100. As CTRL siRNA for all experiments we used a targeting functional siRNA against the gene AHA1, as AHA1 knockdown has no reported effect in hematopoietic cells [2].

Measurement of particle size and Zeta potential

Particle size and Zeta potential were determined using a Zetasizer ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom). For particle size measurements, the LNPs were diluted by a factor of 1 to 10 (50 µl diluted in 450µl buffer) using phosphate-buffered saline. Light scattering measurements were performed at 25 °C in polystyrene cuvettes. For Zeta potential measurements, the LNPs were diluted using phosphate-buffered saline using a dilution factor of 1–100 (20 µl diluted in 450 µl). Measurements were taken thrice for each formulation.

siRNA

The siRNA sequence and details of modifications are as shown in Supplementary Table S1:

Supplementary Table S1.

AHA1_sense	5'-GGAuGAAGuGGAGAuuAGudTsdT -3'
AHA1_antisense	5'-ACuAAUCUCcACUUcAUCCdTsdT -3'
BCR-ABL_unmodified_sense	5'-GCAGAGUUCAAAAGCCCUUdTdT-3'
BCR-ABL_unmodified_antisense	5'-AAGGGCUUUUGAACUCUGCdTdT-3'
BCR-ABL_modification1_sense	5’- gcAGAGuucAAAAgcccuudTsdT -3’
BCR-ABL_modification1_antisense	5’- AAGGGCUUUUGAACUCUGCdTsdT -3’
BCR-ABL_modification2_sense	5’- gcAfGAfgUfuCfaAfaAfgCfcCfuUfdTsdT -3’
BCR-ABL_modification2_antisense	5’- AfAfgGfgCfuUfuUfgAfaCfuCfuGfcdTsdT -3’

Note: a, c, g, u: 2'-O-methyl residues; Af, Cf, Gf, Uf: 2'-F residues; dT: desoxy-T; s: phosphorothioate.

In vivo imaging in mice

The engraftment of luciferase positive cells was measured by bioluminescence imaging on a Xenogen IVIS-200 (Caliper Life Sciences). We imaged the anesthetized animal before the luciferase substrate, luciferin, was given to obtain a background level of light emission. Next, 5 mg/mouse luciferin (P104C, Promega, USA) were injected intraperitoneally. The peak and duration of luciferase activity after intraperitoneal injection of luciferin were determined by IVIS imaging of animals after 7 minutes, with a constant image acquisition time of 1 minute, until a clear peak of light emission was detected. Regions of interest (ROI) were created using Living Image software (Xenogen) on a computer that was dedicated to the IVIS system for data acquisition. These ROIs identify the location of the most intense signal in the bone marrow after delivery of the substrate. An identical ROI (in size and shape) was then created over the luminescent image of the mouse before substrate delivery and photon emission data was measured from this ROI. The photon counts in this control ROI were considered background counts and were subtracted from the experimental data. Data was collected and processed as photons/sec/cm^2 using Living Image software v2.50 (Xenogen).

Inhibition of endocytosis

The role of clathrin and caveolae mediated endocytosis for the uptake of LNP-siRNA formulations was studied in K562 cells. Cells were seeded at 50,000 cells/well in 96-well plates and pre-incubated with inhibitors of endocytosis: chlorpromazine 10µM (clathrin mediated endocytosis), Fillipin 5µM (Caveolae mediated endocytosis) and dynasore 100µM (clathrin and caveolae mediated endocytosis) for 30 minutes. Cells were then treated with DiI labeled LNP-siRNA for 60 minutes in the presence or absence of endocytosis inhibitors. Cells were washed thrice with PBS and the percentage of DiI (LNP) positive cells was analyzed by flow cytometry.

Quantitative Real-Time PCR Primers and Conditions

Total cellular RNA was extracted from cells using TRIzol reagent (Life Technologies). Relative quantification of BCR-ABL mRNA gene expression was carried out with cDNA Reverse Transcription reagents from Applied Biosystems followed by Taqman Gene Expression Assay. The primers and Taqman probe used to detect BCR-ABL are: BCR-ABL_FP-tccactcagccactggatttaa, BCR-ABL_RP- tgaggctcaaagtcagatgctact and BCR-ABL probe- 6-FAM-cagagttcaaaagcccttcagcggc-TAMRA (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany) [3]. The cycling conditions for Taqman PCR include stage 1(50°C for 2 minutes followed by 95°C for 10 minutes) and stage 2 (95°C for 15 seconds and 60°C for 1 minute) for 40 cycles. Relative expression was determined with the $2^{-\Delta\Delta CT}$ method, and the housekeeping gene transcript β-microglobulin (Assay ID: Hs00984230_m1) was used to normalize the results. Experiments were performed in triplicates.

Immunoblotting

Cellular lysates were prepared with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycolate, 0.1% SDS, 100 mM NaF; 10 mM EDTA; 1 mM PMSF; 1 mM Na_2VO4) and protease inhibitor cocktail (Roche, Mannheim, Germany), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane (Whatman GmBH, Dassel, Germany), and immunoblotted with monoclonal mouse anti-ABL antibody from BD Pharmingen (554148), and monoclonal mouse-anti-ß-actin antibody (AC-15 Sigma-Aldrich Hannover, Germany) in 5% BSA overnight at $4^{\circ}C$. Secondary horseradish peroxidase-conjugated antibody used was goat-anti-mouse (Beckman Coulter, Fullerton, CA, USA). Chemiluminescence was used for visualization using the ECL Western blotting detection reagents (Amersham Biosciences-GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Clonogenic progenitor assay

Colony-forming cells (CFCs) were assayed in methylcellulose (Methocult H4100 (StemCell Technologies Inc., Vancouver, Canada)) supplemented with 10 ng/mL human IL3, 10 ng/mL human GMCSF, 50 ng/mL SCF, 50 ng/mL FLT3-ligand and 3 U/mL EPO for human cells. For each round of platings 10^5 human mononuclear cells were plated in duplicate. Colonies were

evaluated microscopically 10 to 14 days after plating by standard criteria. CD34+ cells were incubated with LNP/siRNA for 12 hours in suspension culture in (IMDM medium (12440, Invitrogen) with 10% FCS, 1% Peniciliin/Streptamycin, 200mM L-Glutamine, 0.01M mercaptoethanol and 20ng/ml each of human IL3, human IL6, human GMCSF, human GCSF and human SCF).

Confocal Imaging

For in vitro immunofluorescence analysis, treated cells were fixed in 4% paraformaldehyde at room temperature for 15 minutes, followed by two washes with PBS for 5 minutes. Subsequently, cells were incubated with DAPI solution (100 µg/ml, Sigma-Aldrich, Hannover, Germany) for 5 minutes and were mounted on slides after washing with PBS. Images were acquired using a confocal imaging system (Leica-Inverted-2, Leica Microsystems, Wetzlar, Germany) at 63X objective, using 488 and 561 nm excitation lasers for detection of GFP and DiI, respectively. The pictures were processed with the Leica confocal software (Leica Microsystems).

Patients and culture of primary cells

Primary leukemia cells from bone marrow and peripheral blood of patients with CML were cultured in IMDM medium (12440, Life Technologies) with 10% FCS (Hyclone, Logan, UT), 1% Peniciliin/Streptomycin (PAA laboratories, Pasching, Austria), 200mM L-Glutamine (Thermo Fisher Scientific), 0.01M mercaptoethanol (Sigma-Aldrich) 20ng/ml each of human IL3, human IL6, human GMCSF, human GCSF and human SCF (Peprotech, Rocky Hill, NJ). CD34+ hematopoietic progenitor cells were selected from healthy bone marrow donors. Briefly, mononuclear cells from peripheral blood or bone marrow were isolated by density gradient centrifugation. The cell number was determined and further processing followed the manufacturer's protocol (CD34 microbead kit, Miltenyi Biotech, Bergisch Gladbach, Germany).

Histology of mouse tissues

For histology assessment, LNP-siRNA treated mice were euthanized via CO_2 and then perfused with 4% PFA. Selected tissues were fixed in 4% paraformaldehyde overnight. Tissues were cryo-sectioned at 5 µm and serial histologic sections of the removed hearts, lungs, livers, spleens

and kidneys were stained by DAPI and analysed for LNP-siRNA uptake by immunofluorescence microscope (Olympus BX61).

Clinical chemistry from serum

Clinical chemistry analytes were measured in blood serum. The serum was obtained after centrifugation (13,200 rpm, 5min) and stored at -80 °C until measurement. After manual transfer of the sera into secondary tubes the analyses were carried out on a Cobas® 8000 automated analyzer (Roche Diagnostics, Switzerland) using commercial assays. The assays had been certified for measurement in blood samples. The quantitation of all analytes was done during one run in each sample. Measuring volumes were in the microlitre range. Electrolytes (Na, K, Cl) were determined potentiometrically using ion selective electrodes (ISE). Enzymes, substrates and total protein were measured photometrically using chemical indicator reactions. The detection was based on the consumption of NAD(P)H (ALT, AST, Urea, CK, Glucose, LDH) or on the use of chromogenic substances (Albumin, Alkaline Phosphatase, Amylase, Bilirubine, Creatinine, GGT, Lipase, Total protein). CRP was measured by use of an immuno turbidimetric method. For all measurements, commercial quality control samples were measured several times per day.

Supplemental Figures

Figure S1

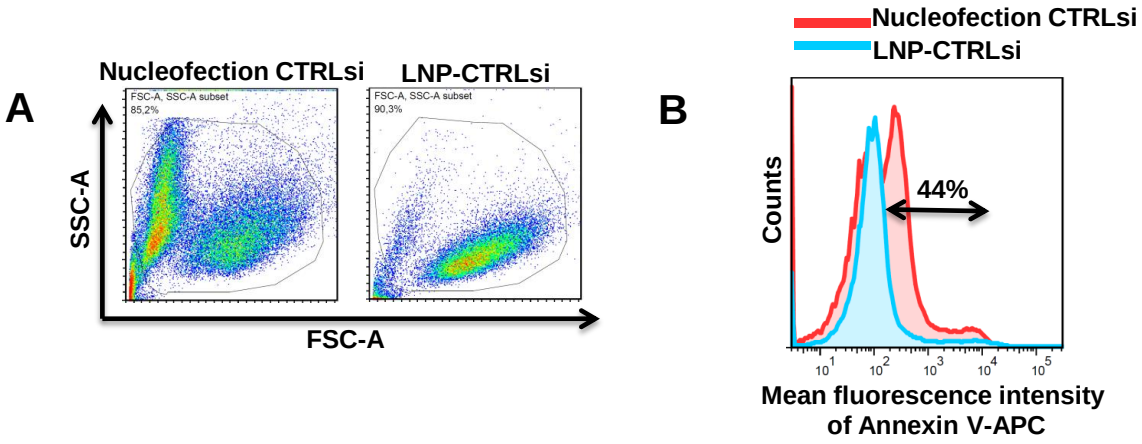

Figure S1. (A) Representative forward and side scatter FACS plot, comparing the effect of nucleofection of CTRL siRNA (290ng) and LNP-CTRL siRNA (290ng) treatment on the viability of K562 cells at 48 hours. (B) Representative FACS plot, comparing the effect of nucleofection of CTRL siRNA (290ng) and LNP-CTRL siRNA (290ng) treatment on the death of K562 cells at 48 hours.

Figure S2

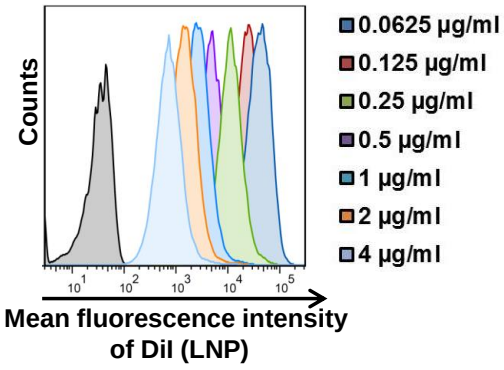

Figure S2. A representative histogram is shown for the dose dependent uptake of DiI (LNP) in K562 leukemic cells at 24 hours following LNP-siRNA treatment at the indicated doses.

Figure S3

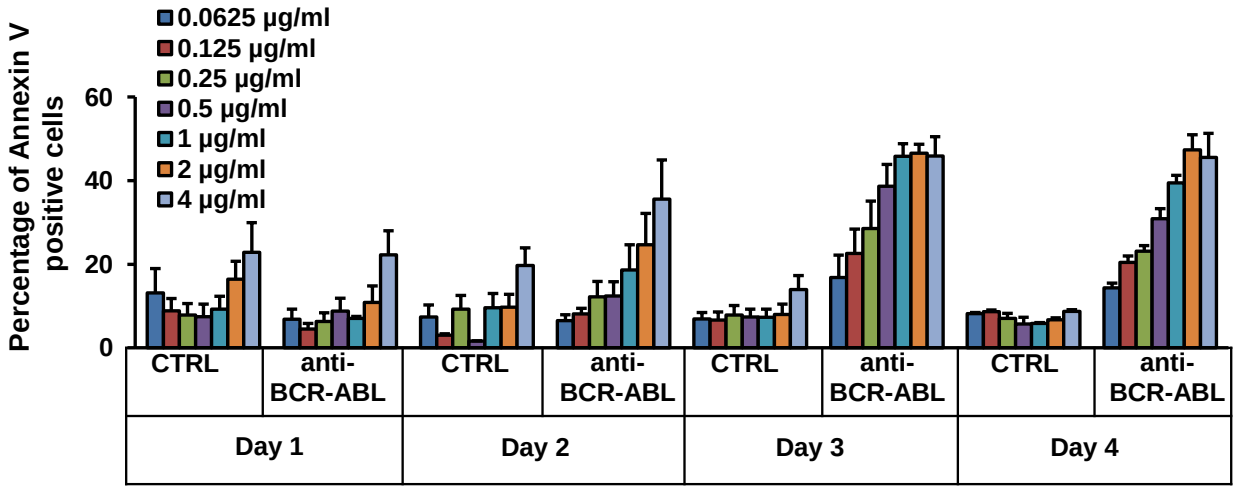

Figure S3. Time and dose dependent induction of apoptosis in K562 cells using anti-BCR-ABL siRNA packaged in LNPs, with indicated concentrations as shown in the figure (mean ± SEM, n=3).

Figure S4

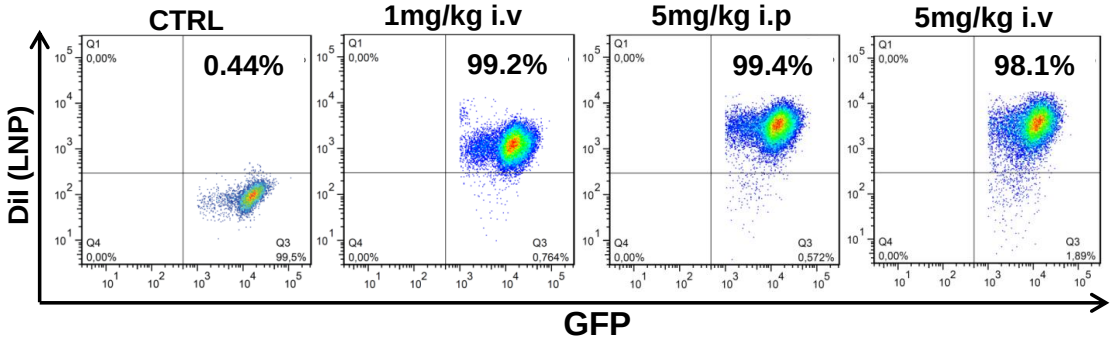

Figure S4. Representative FACS plots of DiI (LNP) positive human leukemic cells in the bone marrow of NSG mice 48 hours after the start of treatment with 3 LNP-CTRL siRNA injections of indicated dose and route of delivery.

Figure S5

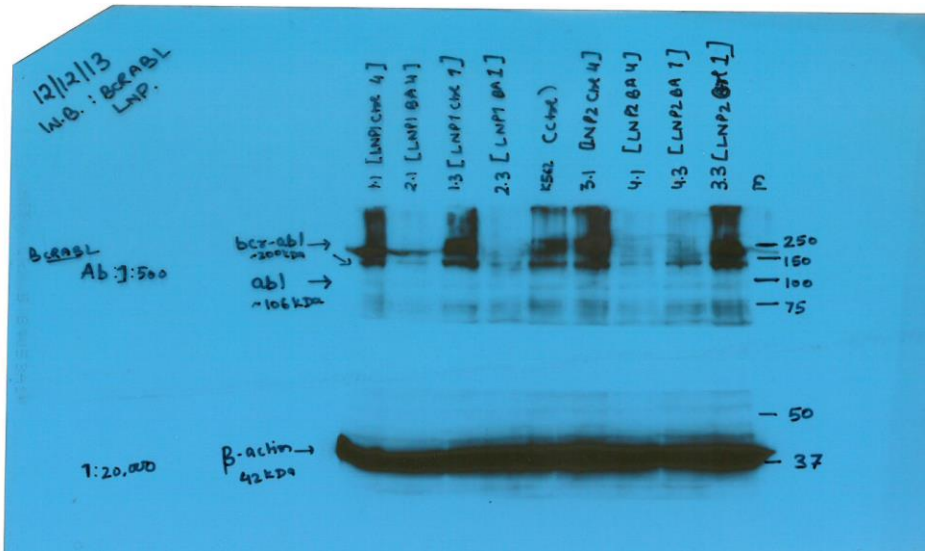

Figure S5. Original western blot showing knockdown of BCR-ABL protein expression in K562L.GFP cells treated with LNP CTRL or anti-BCR-ABL siRNA for 96 hours.

Supplemental References

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2. Dohmen C, Frohlich T, Lachelt U, Rohl I, Vornlocher HP, Hadwiger P, Wagner E (**2012**). Defined Folate-PEG-siRNA Conjugates for Receptor-specific Gene Silencing. **Mol Ther Nucleic Acids** 1(e7. doi: 10.1038/mtna.2011.10.

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