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RNA interference efficiently targets human leukemia driven by a fusion oncogene in vivo

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Despite the wide therapeutic potential of RNA interference (RNAi), clinical progress has been slow with only a few examples of successful translation. Efficient knockdown of hepatic transthyretin (87%) in patients with transthyretin amyloidosis lasted for several weeks after a single dose.1 Also, in a phase I clinical trial, a single dose of inclisiran (siRNA against the *PCSK9* mRNA) efficiently suppressed serum cholesterol for 6 months.2 However, these studies suggested that siRNA delivery beyond the liver is not yet feasible in the clinic and thus limits the potential benefit of RNAi. Lipid nanoparticles (LNPs) containing ionizable cationic lipids embody the most advanced delivery platform for systemic administration of RNAi therapeutics.3 Our study provides a preclinical proof-of-

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Conflict of interest

Euan Ramsay is an employee of Precision Nanosystems. Pieter Cullis is founder of Precision Nanosystems. The other authors have no conflicts of interest.

concept that RNAi therapeutics can be exploited against leukemia cells using LNPs as a delivery tool, in a patient derived B cell acute lymphoblastic leukemia (ALL) xenograft mouse model.

Chromosomal translocations are considered driver mutations in leukemogenesis, and are usually present in all leukemic cells and are retained during relapse.[4] 75% of pediatric ALL patients harbour gross chromosomal aberrations, with *ETV6-RUNX1*, *TCF3-PBX1*, *MLL* rearrangements and *BCR-ABL1* being the most relevant mutations.[5] Continuous efforts have been made to target fusion oncogenes by delivering siRNAs in vitro such as anti-BCR-ABL siRNA in K562 cells,[6] and anti-MLL-AF9 siRNA in THP1 cells,[7] and in vivo such as anti-SS18-SSX1 siRNA in a synovial sarcoma xenograft model[8] and anti-TMPRSS2-ERG siRNA in a prostate cancer xenograft model.[9] Anti-BCR-ABL siRNA was also administered intravenously in an imatinib resistant chronic myeloid leukemia (CML) patient and showed efficient knockdown of the BCR-ABL fusion gene and good tolerability.[10]

The translocation t(1;19)(q23;p13), resulting in the fusion gene *TCF3-PBX1* (also called *E2A/PBX1*), is one of the most frequent translocations in B-ALL in both adult and pediatric populations at an overall frequency of 5-10%.[11] Despite of intensive chemotherapy approximately 10% of *TCF3-PBX1* positive patients experience relapse with dismal prognosis, and novel treatment approaches are urgently needed for these patients.[12]

To improve delivery of siRNAs to non-hepatic tissues, especially to leukemic cells in bone marrow, spleen and blood, we developed proprietary lipid nanoparticles (LNP, SUB9KITS, see Supplemental Methods) and a microfluidics device (NanoAssemblr™) to reproducibly encapsulate siRNA in LNPs (Supplemental Figure S1A). LNP-siRNA intracellular uptake and efficacy were evaluated in vitro in *TCF3-PBX1* expressing 697 cells (DSMZ, Braunschweig, Germany) and in vivo in a patient derived xenograft (PDX) model from a *TCF3-PBX1* positive ALL patient.[13] Packaged LNPs were analyzed for size and charge characteristics with the zetasizer instrument (Malvern Instruments, Herrenberg, Germany). The estimated mean diameter of our LNP-siRNA formulations (lipid/siRNA weight ratio 10:1) was 55 nm (Supplemental Figure S1B). We reproducibly encapsulated more than 90% of the used siRNA inside the LNPs (Supplemental Figure S1C).

The efficacy of four manually designed siRNAs covering the fusion point of *TCF3-PBX1* was evaluated in 697 cells in vitro. Anti-TCF3-PBX1 siRNA3 was most effective (87% knockdown) and was used for all further experiments (Supplemental Figure S2). The delivery efficiency at various concentrations (0.25-2μg/ml) of LNP-siRNA formulations in 697 cells was 100% even at the lowest concentration (Figure 1A). The LNP-*TCF3-PBX1* siRNA efficiency in 697 cells was confirmed by RT-PCR showing a knockdown of *TCF3-PBX1* expression up to 80% at higher and 65-70% at lower concentrations compared to LNP-CTRL siRNA at 72 hours (Figure 1B). Also, we detected a robust knockdown of the TCF3-PBX1 protein as shown by Western blot at 72 hours in 697 cells treated with LNP-siRNA (CTRL or TCF3-PBX1, Supplemental Figure S3A). 697 cells underwent cell death in a concentration dependent manner when treated with LNP-TCF3-PBX1 siRNA but not with LNP-CTRL siRNA (Figure 1C). Consistently, we observed a significant increase in apoptotic Annexin V positive cells treated with LNP-TCF3-PBX1 siRNA compared to LNP-

CTRL siRNA (Supplemental Figure S3B). To confirm the specific nature of TCF3-PBX1 siRNA, we also treated the K562 cell line with LNP-TCF3-PBX1 siRNA formulations and did not observe any significant non-specific effects on cell viability and apoptosis (Supplemental Figure S4A-B). Moreover, no significant decrease in expression levels of *TCF3* and *PBX1* mRNA levels in K562 cells were observed (Supplemental Figures S4C-D).

To evaluate the LNP-siRNA uptake in difficult to transfect human patient derived cells, we incubated the LNP-CTRL siRNA formulation with freshly isolated leukemic cells from patients with CML and ALL in cytokine supplemented primary human cell culture media. We observed an efficient dose dependent uptake of LNP-CTRL siRNA in the CD34 positive population and with variable efficacy in CD34 negative primary cells from these patients at 72 hours after a single treatment (Supplemental Figure S5A-B). These data show that our LNPs are efficiently taken up under normal growth conditions even in difficult to transfect primary myeloid and lymphoid leukemic cells. In order to confirm the internalization of LNP-siRNA, we performed confocal microscopy in primary cells of a patient with acute myeloid leukemia after treatment with 1μg/ml of LNP-CTRL siRNA or LNP-fluorescein isothiocyanate (FiTC) tagged-CTRL siRNA. We quantified the confocal images and found that all cells treated with LNP-FiTC tagged siRNA showed cytoplasmic uptake of the siRNAs (Supplemental Figure S5C for confocal images and Figure S5D for quantification of the FiTC signal).

We next assessed the delivery potential and efficacy of our LNPs *in vivo*, with a focus on hematopoietic tissues following systemic administration. Female 6-8 week old Nod-Scid-IL2Rgamma^{null} (NSG) mice transplanted intrafemorally with K562 leukemia cells received 3 injections of LNP-CTRL siRNA at different doses (1 or 5 mg/kg body weight) and routes of administration (intravenously (i.v.) or intraperitoneally (i.p.)). The 3 injections were applied at 0, 8 and 24 hours starting 10 days after transplantation, and mice were analyzed at 48 hours. Importantly, 89-95% of human K562 cells had taken up LNPs in myelosarcoma tissue and 67-99% in murine cells from different organs (peripheral blood, bone marrow, spleen, liver) independent of the routes of administration (Supplemental Figure S6). The percentage of LNP positive cells was significantly lower in all organs except myelosarcoma tissue at a dose of 1 mg/kg compared to a dose of 5 mg/kg (Supplemental Figure S6).

To evaluate our technology in primary patient cells in vivo,13 we transplanted cells from the TCF3-PBX1 positive B-ALL patient in sub-lethally irradiated NSG mice. We treated the TCF3-PBX1 dependent B-ALL PDX mice with 10 injections of 2.5mg/kg LNP-CTRL siRNA or LNP-TCF3-PBX1 siRNA, starting from day 7 after transplantation over a period of 24 days. The majority of human (and mouse) cells in the bone marrow of treated mice showed uptake of LNPs at death (48 hours after the last injection, Supplemental Figure S7A). We observed a 55% knockdown of *TCF3-PBX1* mRNA levels in spleen cells from moribund mice treated with 10 injections of LNP-TCF3-PBX1 siRNA compared to LNP-CTRL siRNA (Figure 2A). The leukemia development in mice was monitored by quantifying the percentage of CD45+ transplanted human leukemia cells in peripheral blood. A delayed onset of leukemia and significantly lower engraftment of CD45+ cells were observed in mice treated with LNP-TCF3-PBX1 siRNA compared to mice treated with LNP-CTRL siRNA (Figure 2B). Importantly, mice treated with LNP-TCF3-PBX1 siRNA survived significantly longer compared to LNP-CTRL siRNA treated mice (median OS 45 days vs 32 days, P=0.0026, Figure 2C). At day 33, white blood cell counts (WBC) were significantly lower and higher platelet counts were observed in LNP-TCF3-PBX1 siRNA treated mice (Supplemental Figures S7B-C). In the bone marrow of moribund mice treated with LNP-TCF3-PBX1 siRNA, we found a lower proportion of blast cells than in LNP-CTRL siRNA treated mice (Supplemental Figures S7D and S7E). By targeting the *TCF3-PBX1* fusion oncogene we show a reduction of leukemic burden in our patient derived lymphoblastic leukemia xenotransplant mouse model, and demonstrate improved survival of PDX mice treated with LNP-TCF3-PBX1 siRNA as compared to LNP-CTRL siRNA.

It has been shown that the intracellular uptake of our nanoparticles is dependent on the association with ApoE and binding to the low density lipoprotein receptor (LDL).3 The LDL receptor is widely expressed on leukemic cells and was also expressed in our TCF3-PBX1 positive leukemia cells (Supplemental Figure S8A-B). So far, no cellular ligand has been identified that is selectively expressed on leukemic but not on normal stem cells. Effective inhibition of cyclin D1 in a mantle cell lymphoma mouse model using αCD38 antibody-LNPs encapsulating CycD1 siRNA was recently reported by Peer et al.14 However, conjugating a targeting ligand to delivery systems may result in physicochemical instability in blood circulation and decreased accumulation at target tissues.15 Thus, the use of a leukemia-specific siRNA enabled us to abstain from a targeted delivery approach. In summary, we have developed LNP-siRNA formulations that target primary human leukemia cells *in vitro* and *in vivo* with high efficacy, deliver a leukemia-specific siRNA to leukemic cells and thus prolong survival of mice bearing a patient derived TCF3-PBX1 positive ALL. Fusion oncogenes thus represent disease specific targets for RNAi and should be exploited to realize a new mode of personalized treatment in leukemia patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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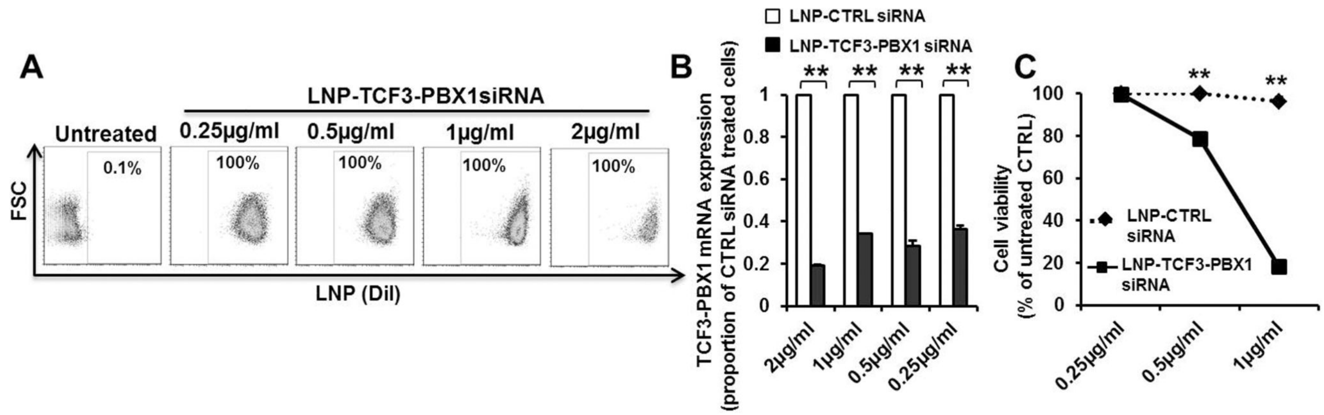

Figure 1. Uptake and on-target efficacy of LNP-siRNA formulations in TCF3-PBX1 expressing 697 human B-ALL leukemia cells in vitro.

A. Representative FACS plot showing the percentage of Dil (LNP) positive cells as measured by flow cytometry in TCF3-PBX1 expressing 697 cells treated with LNP-TCF3-PBX1 siRNA (0.25, 0.5, 1 and 2 μg/ml siRNA, corresponding to 17.85, 35.7, 71.43, and 143 nM siRNA, respectively) after 72 hours. The concentrations in μg/ml refer to the siRNA concentration.

B. RT-PCR validation of TCF3-PBX1 knockdown in the TCF3-PBX1 positive 697 cell line in vitro using LNP encapsulated TCF3-PBX1 siRNA or LNP-CTRL siRNA after 72 hours of treatment (mean ± SEM, n=3).

C. Viability of 697 cells treated in vitro with LNP-CTRL or LNP-TCF3-PBX1 siRNA for 6 days at the indicated concentrations (mean ± SEM, n=6).

** indicates P<.01.

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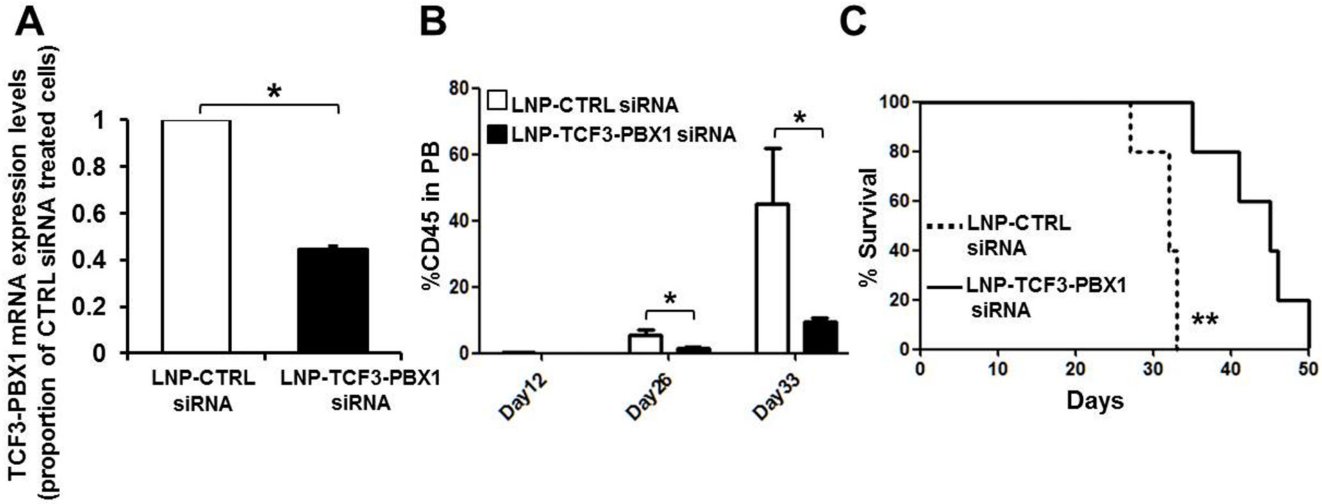

Figure 2. TCF3-PBX1 siRNA can be efficiently delivered in patient derived human acute lymphoblastic leukemia cells in vivo in a xenograft mouse model and prolongs survival of mice.

A. Knockdown of TCF3-PBX1 in spleen cells of TCF3-PBX1 expressing ALL-PDX mice treated with LNP-siRNA (CTRL or TCF3-PBX1). LNP-siRNA formulations (2.5mg/kg) were injected intraperitoneally 10 times (days 7, 8, 13, 15, 19, 20, 22, 23, 29 and 30, considering the transplantation date as day 0) (mean ± SEM, n=3).

B. Engraftment of human CD45 positive primary ALL cells at different time points in peripheral blood (PB) of TCF3-PBX1 expressing ALL-PDX mice treated with LNP-siRNA (CTRL or TCF3-PBX1, n=5 each). LNP-siRNA formulations (2.5mg/kg) were injected 10 times (days 7, 8, 13, 15, 19, 20, 22, 23, 29 and 30, considering the transplantation date as day 0).

C. Survival of ALL-PDX mice treated with LNP-siRNA (CTRL or TCF3-PBX1). LNP-siRNA formulations (2.5mg/kg) were injected 10 times (days 7, 8, 13, 15, 19, 20, 22, 23, 29 and 30, considering the transplantation date as day 0; n=5 per group).

* indicates P<.05, ** indicates P<.01, ns, not significant.