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LETTERS TO THE EDITOR

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] Furthermore, in a phase I clinical trial, a single dose of inclisiran (small interfering RNA (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-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 that are usually present in all leukemic cells and are retained during relapse.[4] Seventy five percent of pediatric ALL patients harbor gross chromosomal aberrations, with *ETV6-RUNX1*, *TCF3-PBX1*, *MLL* rearrangements and *BCR-ABL1* being the most relevant translocations.[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 (i.v.) in an imatinib resistant chronic myeloid leukemia (CML) patient and showed efficient knockdown of the *BCR-ABL* fusion gene with 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 with an overall frequency of 5–10%.[11] Despite of intensive chemotherapy, ~10% of *TCF3-PBX1*-positive patients experience relapse with dismal prognosis and novel treatment approaches are urgently needed for such patients.[12]

To improve delivery of siRNAs to non-hepatic tissues, especially to leukemic cells in the bone marrow, spleen and blood, we developed proprietary LNPs (SUB9KITS, see Supplementary Methods) and a microfluidics based device (NanoAssemblr, Precision Nanosystems, Vancouver, Canada) to reproducibly encapsulate siRNA in LNPs (Supplementary 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 (Supplementary Figure S1B). We reproducibly encapsulated >90% of the used siRNA inside the LNPs (Supplementary 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 (Supplementary Figure S2). The delivery efficiency at various concentrations (0.25–2 μg/ml) of LNP-siRNA formulations in 697 cells was 100% even at the lowest concentration (Figure 1a). The LNP-*TCF3-PBX1* siRNA efficiency

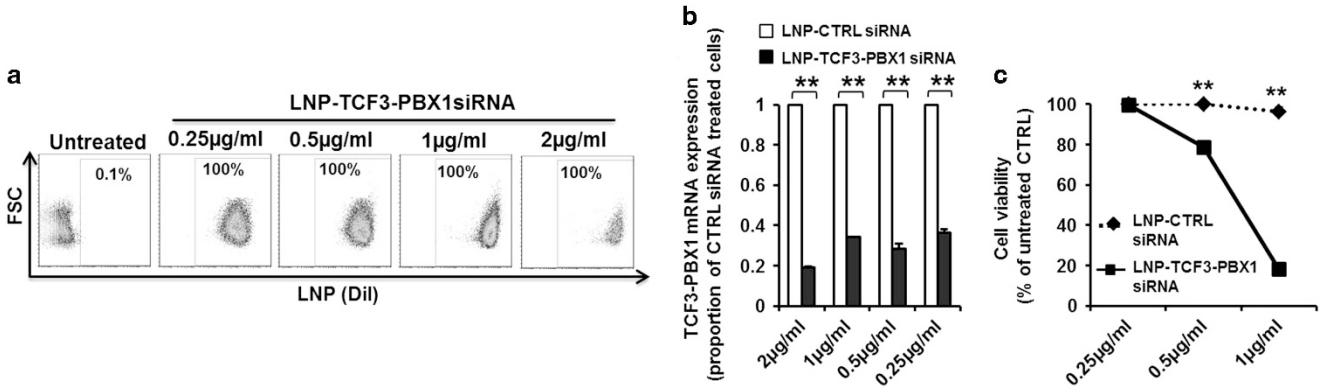

Figure 1. Uptake and on-target efficacy of LNP-siRNA formulations in *TCF3-PBX1*-expressing 697 human B-ALL leukemia cells *in vitro*. (**a**) Representative fluorescence-activated cell sorting (FACS) plot showing the percentage of Dil (LNP)-positive cells as measured by flow cytometry in *TCF3-PBX1*-expressing 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 h. The concentrations in μg/ml refer to the siRNA concentration. (**b**) Real time quantitative (RT)-PCR validation of *TCF3-PBX1* knockdown in the *TCF3-PBX1*-positive 697 cell line *in vitro*, treated with LNP-encapsulated TCF3-PBX1 siRNA or LNP-CTRL siRNA after 72 h of treatment (mean ± s.e.m., $n=3$). (**c**) Viability of 697 cells treated *in vitro* with LNP-CTRL or LNP-TCF3-PBX1 siRNA for 6 days at the indicated concentrations (mean ± s.e.m., $n=6$). $^{**}P<.01$.

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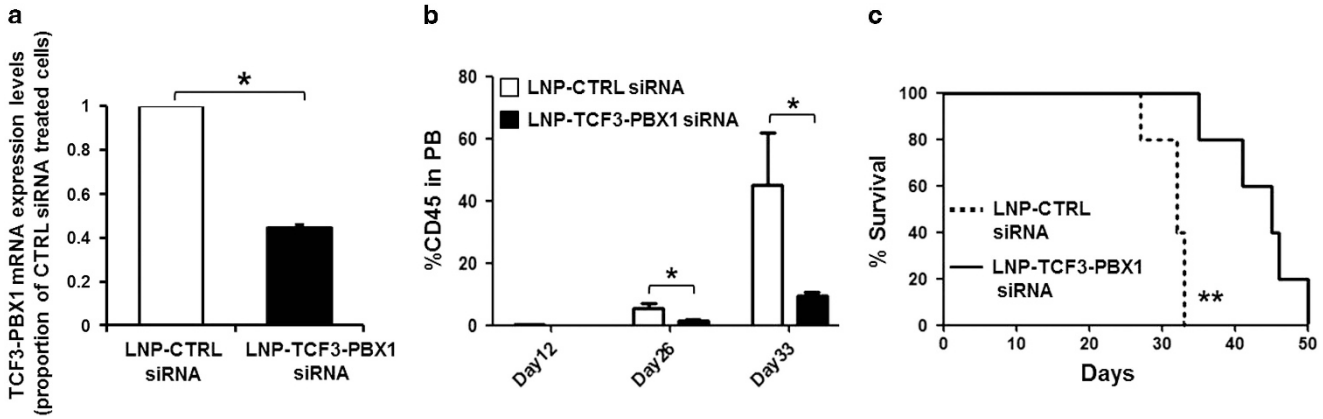

Figure 2. TCF3-PBX1 siRNA can be efficiently delivered in patient-derived human ALL cells *in vivo* in a xenograft mouse model and prolongs survival of mice. (**a**) Knockdown of *TCF3-PBX1* in spleen cells of *TCF3-PBX1*-expressing ALL-PDX mice treated with LNP-siRNA (CTRL or TCF3-PBX1). LNP-siRNA formulations (2.5 mg/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 ± s.e.m., $n = 3$). (**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 (2.5 mg/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 (2.5 mg/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). *$P < .05$, **$P < .01$, ns, not significant.

in 697 cells was confirmed by reverse-transcriptase PCR showing a knockdown of *TCF3-PBX1* expression up to 80% at higher and 65–70% at lower concentrations compared with LNP-CTRL siRNA at 72 h (Figure 1b). In addition, we detected a robust knockdown of the TCF3-PBX1 protein as shown by western blotting at 72 h in 697 cells treated with LNP-siRNA (CTRL or TCF3-PBX1, Supplementary 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 with LNP-CTRL siRNA (Supplementary Figure S3B). To confirm the specific nature of TCF3-PBX1 siRNA, we also treated the K562 leukemia cell line with LNP-TCF3-PBX1 siRNA formulations and did not observe any significant non-specific effects on cell viability and apoptosis (Supplementary Figures S4A and B). Moreover, no significant decrease in expression levels of *TCF3* and *PBX1* mRNA levels in K562 cells were observed (Supplementary Figures S4C and 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 h after a single treatment (Supplementary Figures S5A and B). These data show that our LNPs are efficiently taken up under normal growth conditions even in difficult to transfect primary myeloid and lymphoid leukemia 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 1 µg/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 (Supplementary Figure S5C for confocal images and Supplementary 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. 6–8-week-old Nod-Scid-IL2Rgamma^{null} female (NSG) mice transplanted intrafemorally with K562 leukemia cells received three injections of LNP-CTRL siRNA at different doses (1 or 5 mg per kg body weight) and routes of administration (intravenously or intraperitoneally). The three injections were applied at 0, 8 and 24 h starting 10 days after transplantation and mice were analyzed at 48 h. 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 and liver) independent of the routes of administration (Supplementary 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 with a dose of 5 mg/kg (Supplementary Figure S6).

To translate our findings 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 2.5 mg/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 h after the last injection, Supplementary 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 with LNP-CTRL siRNA (Figure 2a). The leukemia development in mice was monitored by quantifying the percentage of CD45+ transplanted human leukemia cells in the 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 with mice treated with LNP-CTRL siRNA (Figure 2b). Importantly, mice treated with LNP-TCF3-PBX1 siRNA survived significantly longer compared with LNP-CTRL siRNA treated mice (median OS 45 days vs 32 days, $P = 0.0026$, Figure 2c). At day 33, white blood cell counts were significantly lower and higher platelet counts were observed in LNP-TCF3-PBX1 siRNA treated mice (Supplementary Figures S7B and 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 (Supplementary Figures S7D and E). 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 with 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 (LDL) receptor.[3] The LDL

receptor is widely expressed on leukemic cells and was also expressed in our TCF3-PBX1-positive leukemia cells (Supplementary Figures S8A and 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.

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.

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