Rational design of an immunoconjugate for selective knock-down of leukemia-specific E2A–PBX1 fusion gene expression in human Pre-B leukemia

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The t(1;19)(q23;p13) is one of the most common chromosomal translocations in acute lymphoblastic leukemia (ALL) and results in production of the transforming oncoprotein E2A–PBX1. Here we first report a novel, biomarker-guided biotherapy strategy for personalized treatment of t(1;19)+ ALL. A supervised interrogation of the gene expression profiles of primary leukemic cells from a cohort of 207 children with high risk B-lineage ALL identified up-regulated CD19 gene expression as a biomarker for t(1;19)+ ALL. A disulfide-linked immunoconjugate of a 5-amino-modified 24 mer phosphorothioate anti-sense E2A–PBX1 oligonucleotide (AON) with a mAb specific for a CD19 receptor (αCD19–AON) was prepared as a CD19-directed and leukemia-specific biotherapeutic agent against E2A–PBX1+ B-lineage ALL. Treatment of E2A–PBX1+ leukemia cells with low nanomolar concentrations of αCD19–AON resulted in selective depletion of E2A–PBX1 transcripts and caused apoptotic destruction and abrogation of clonogenic growth. Subcutaneously administered αCD19–AON at a total dose level of 93 nmol kg−1 delivered over 14 days using a micro-osmotic pump more than doubled the leukemia-free survival time of SCID mice in a xenograft model of E2A–PBX1+ human B-lineage ALL (82.0 ± 1.9 days vs. 37.0 ± 0.1 days, P < 0.0001). Both the AON moiety and the targeting CD19-specific mAb moiety were required for the in vitro as well as in vivo anti-leukemic activity of αCD19–AON. The observed in vitro and in vivo anti-leukemic potency of the αCD19–AON immunoconjugate provides the first preclinical proof-of-principle that t(1;19)+ high risk B-lineage ALL can be treated with leukemia-specific biotherapeutic agents that knock-down E2A–PBX1 expression.

Introduction

The transcription factor 3 gene (TCF3/E2A) located on the short arm of chromosome 19 at band 19p13.3 encodes two transcription factors, E12 and E47, both of which belong to the class I family of basic helix-loop-helix (bHLH) proteins.1 In B-cell ontogeny, E2A homodimeric complexes exert pivotal regulatory functions, including activation of B-cell specific gene expression and immunoglobulin gene rearrangement.2,3 Targeted disruption of the E2A locus in mice results in a maturational arrest of B-lineage lymphoid progenitor cells at the early pro-B cell stage prior to the onset of Ig heavy chain rearrangement.2,3 The chromosomal translocation t(1;19)(q23;p13.3), the second most common translocation in acute lymphoblastic leukemia (ALL) and the most common TCF3/E2A gene rearrangement,4–7 results in the expression of a nuclear
oncoprotein, which is comprised of the N-terminal transactivation domains of E2A fused with the C-terminal DNA binding homedomain of PBX1 (pre-B cell leukemic homeobox1) encoded by PBX1 at 1q23.7–9 This chimeric E2A–PBX1 fusion protein acts as a transcriptional activator and is capable of cellular transformation.8–10 A number of genes specifically expressed in t(1;19)+ ALL cell lines were identified, including Wnt16 that encodes a potentially leukemogenic growth factor of the Wnt signaling pathway capable of stimulating proliferation of B-cell precursors.11 t(1;19)+ ALL is an immunophenotypically and prognostically distinct subset of B-lineage ALL that is characterized by expression of cytoplasmic immunoglobulin in leukemia cells (pre-B cell stage of differentiation), specific clinical and biological features and a signature transcriptome.12–16 Although the overall prognostic significance of t(1;19) has been obviated by contemporary risk-adjusted protocols, the balanced t(1;19) translocation remains an adverse prognostic factor.12,14 The purpose of the present study was to evaluate the clinical potential of a new biotherapeutic strategy against t(1;19)+ ALL that employs a rationally designed immunoconjugate for selective knockdown of the E2A–PBX1 fusion transcript expression in pre-B ALL cells.

Materials and methods

Bioinformatics and statistical analysis of gene expression profiles

Gene pattern [http://www.broadinstitute.org/cancer/software/genepattern]17 was used to extract expression values for 11 genes; CD19, CD2, CD22, CD34, CD40, CD5, CD72, IGF1R, IL2RA, IL3RA, IL7R, obtained from matched pair bone marrow specimens of ALL patients at the time of initial diagnosis (1st specimen) and then at first relapse. Matched pair expression values were taken from 59 B-lineage ALL patients at diagnosis and then at relapse combined from GSE3912 (N = 32)18 and GSE18497 (N = 27).19 To determine the differential expression of each gene, paired t-tests were performed for the combined mean centered values from GSE3912 and GSE18497 datasets (unequal variance correction, P < 0.05 deemed significant). Comparison of early (N = 40; <36 months) versus late (N = 19; ≥36 months) relapse subsets for newly diagnosed patients was performed to identify potential biomarkers for early relapse (2-sample t-test, unequal variances). The expression of this gene set was also calculated in samples obtained from 23 E2A–PBX1 positive patients in a cohort of 207 patients with high risk B-lineage ALL (COG P9906, GSE11877, MAS5.0 normalized expression values,20) (t-test, unequal variances, P < 0.05 deemed significant). We used a two-way agglomerative hierarchical clustering technique to organize expression patterns using the average distance linkage method such that gene transcripts (rows) and samples (columns) having similar expression values were grouped together (calculated using the average distance metric). Dendrograms were drawn to illustrate similar gene-expression profiles from joining pairs of closely related gene expression profiles, whereby genes and samples joined by short branch lengths showed most similarity in expression profiles across patient samples and genes (JMP Software, SAS, Cary, NC). The heat map represents the color-coded expression value reported as a mean centered expression level relative to log10 transformed diagnostic samples.

Preparation of an αCD19–AON immunoconjugate

The two-step method that was used for immunoconjugation involved modification of the mAb and AON moieties prior to their incubation to prepare a disulfide-linked mAb–AON conjugate. 24 mer oligonucleotide (ON) phosphorothioates were obtained from the Midland Certified Reagent Company (Midland, Texas). Each ON contained a 6-carbon amino modification at its 5-terminus. The antisense ON (AON) had a sequence complementary to the sequence of the E2A–PBX1 fusion transcript: 5’-GAT ACT CAA AAC ACT GTA GGA GTC-3’. The scrambled ON (SON) consisted of the same number of all 4 bases that were arranged randomly: 5’-TGA GCA GTA GAT AGT ACG TAA CCC AAT-3’. Approximately 2.8 μmol (22 mg) of each amino-modified ON were reacted for 3 h at room temperature with the thiolating agent 2-iminothiolane (2-IT) (30 mM solution in 50 mM dibasic sodium phosphate, pH 8.6) (Pierce Chemical Co., Rockford, IL) at a 50 : 1 molar ratio (2-IT : ON) to generate an amide compound that has a free sulfhydryl group. The mixture was concentrated by centrifugation through a Centricon-3 device (Amicon, Beverly, MA) to remove excess 2-IT prior to passage through a prepaked PD-10 column (Pharmacia Biotech, Piscataway, NJ). Purified αCD19–mAb21 (8 mg ml−1 in PBS, pH 7.5) was modified with N-succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) (Pharmacia) at a 25 : 1 molar ratio of SPDP : mAb, as previously reported.22 The amine-reactive portion of SPDP is the N-hydroxysuccinimide (NHS) ester. The sulfhydryl-reactive portion of SPDP is the 2-pyridylidithio group, which reacts optimally with sulfhydryl groups between pH 7 and 8.1. SPDP was dissolved in DMSO at a concentration of 190 mM prior to use. After a 3 h reaction at room temperature, excess SPDP was removed by passage through a PD-10 column equilibrated in PBS. Fractions containing the majority of the SPDP-modified/pyridyldithiol-activated αCD19–mAb protein were concentrated to 8 mg ml−1 using Centricon-30 centrifugal concentrating devices (Amicon) and mixed with an equal volume of the derivatized AON at a final molar ratio of 15 : 1 (AON : mAb) to prepare a disulfide-linked mAb–AON conjugate. The mixture was gently rotated overnight at room temperature and concentrated 2-fold prior to being filtered (0.2 μm) and injected into a Beckman System Gold HPLC TSKG3000SW 21.5 × 600 mm size exclusion column (TosoHaas, Montgomeryville, PA) to separate unreacted AON from mAb–AON conjugates. The column was equilibrated in 100 mM sodium phosphate buffer, pH 6.8 at a flow rate of 3 ml min−1. The mAb–AON immunoconjugate eluting at 30–40 min post-injection was collected and concentrated.

Binding and internalization studies

8 mg (~1 μmol) of the AON were tritiated at the C-8 thymidine position by ChemSyn Laboratories (Lenexa, Kansas). 680 μCi of 3H-AON were obtained and had a specific activity of 0.085 μCi μg−1 (6.6 × 105 cpm nmol−1). 5 mg of this radiolabeled AON were linked
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Confocal laser scanning microscopy

Confocal microscopy was performed using previously published procedures. Leukemia cells treated with CD19-AON or control reagents CD7-AON, CD19-SON, and unconjugated CD19-mAb for 96 h at 37 °C/5% CO₂ were attached to poly-L-lysine-coated coverslips and fixed in ice-cold (-20 °C) methanol for 15 min. After fixation, the coverslips were washed for 15 min in phosphate-buffered saline (PBS) + 0.1% Triton X-100. Cells were stained with a rabbit polyclonal anti-tubulin antibody according to the manufacturer’s recommendations (Sigma, St. Louis, MO, USA) to visualize their cytoplasms. The secondary antibody was a goat anti-rabbit fluorescein-conjugated antibody. DNA was labeled for 15 min with 1 μM Toto-3, a DNA specific dye (Molecular Probes, Eugene, OR) to visualize the apoptotic changes in the nuclei. Coverslips were inverted and mounted onto slides in Vectashield (Vector Labs, Burlingame, CA) to prevent photobleaching and were sealed with nail varnish. Slides were examined using a Bio-Rad MRC-1024 laser scanning confocal microscope mounted on a Nikon Eclipse E-800 upright microscope equipped for epifluorescence with high numerical aperture objectives.

Clonogenic assays and statistical analysis using planned linear contrasts

Leukemic cell lines were treated with various controls and test agents and then assayed for colony formation in a semi-solid methylcellulose culture system as previously described. Controls included samples exposed to 4 Gy γ-rays or 25 μg ml⁻¹ vincristine (VCR), a standard chemotherapy drug used in ALL therapy. In brief, treated cells (10⁵ cells ml⁻¹) were washed twice, and then suspended in alpha-MEM supplemented with 0.9% methylicellulose, 30% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Controls included untreated cells. Duplicate 1 ml samples containing 10⁴ cells per sample were cultured in 35 mm Petri dishes for 7 days at 37 °C in a humidified 5% CO₂ atmosphere. On day 7, colonies containing ≥ 30 cells were counted using an inverted phase microscope with high optical resolution. We constructed 4 General Linear Models for each cell type (LC1;19, RS4;11, ALL-1, MOLT3) with two factors ("treatment" and "experiment (2 replicates per experiment)") for the analysis of the clonogenic death assay data expressed as colony count (mean no. colonies per 10⁴ cells plated after test treatment)/(mean no. colonies per 10⁴ cells plated after vehicle control treatment). One factor for the dependent variable was the “treatment” (9 levels for LC1;19 and 4 levels for RS4;11, ALL-1, MOLT3) with two factors ("treatment" and "experiment (2 replicates per experiment)" for the analysis of the clonogenic death assay data expressed as colony count (mean no. colonies per 10⁴ cells plated after test treatment)/(mean no. colonies per 10⁴ cells plated after vehicle control treatment). One factor for the dependent variable was the “treatment” (9 levels for LC1;19 and 4 levels for RS4;11, ALL-1, MOLT3) with two factors ("treatment" and "experiment (2 replicates per experiment)" for the analysis of the clonogenic death assay data expressed as colony count (mean no. colonies per 10⁴ cells plated after test treatment)/(mean no. colonies per 10⁴ cells plated after vehicle control treatment).
(2 levels) to consider 2 sources of variation for the treatment effect arising from replicates within an experiment and variation between experiments. Consistent treatment effects for 4 replicates partitioned across 2 experiments were accounted for by normalizing differences between experiments utilizing the variance component in the “experiment” factor. In this design, differences in treatment effects were discerned in cases where there were significant differences in the overall mean of colony counts for all treatments and replicates between the 2 experiments. The least squares method was used to fit the parameters for the General Linear Models (model for LC1;19 included an intercept, 8 parameters for the treatment factor and 1 parameter for the experiment factor, and the model for the other 3 cell lines included an intercept, 3 parameters for the treatment factor and 1 parameter for the experiment factor). Model parameters were utilized to generate prediction equations and best fit lines visualized by plotting Leverage graphs using standard coding procedures. We examined the distribution of the residuals of the model for equal dispersion around the line of best fit that compared predicted and observed cell death values. The root mean square error term calculated from the deviation of data points from the model was utilized to determine significant treatment effects in the planned linear contrasts (JMP Software, SAS, Cary, NC). These contrasts compared the effect sizes such that the combination of linear parameters to be jointly tested sum to zero for each level of the contrast. Least square mean values were used to construct contrasts between each of the treatment groups (set to −1) and the control group (set to 1) for each cell type. Two-tailed tests for differences between the least square means with P-values less than 0.05 were deemed statistically significant. Post-hoc statistical power analysis for the General Linear Models was also performed for cell death assays at the 5% significance level (JMP Software, SAS, Cary, NC).

SCID mouse xenograft model of t(1;19) ALL

Mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC), and all animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington DC 1996). LC1;19 is an E2A–PBX1+ B-lineage ALL cell line established from leukemic SCID mouse xenografts of primary leukemic cells from a t(1;19)− ALL patient who relapsed at 8.8 months. It causes rapidly progressive, disseminated and invariably fatal leukemia in SCID mice. Female C.B.17 SCID mice (6–8 weeks of age; Taconic/Germantown, NY) were inoculated intravenously with 0.2 ml of a cell suspension containing 1 × 10⁶ LC1;19 cells, as previously reported. On the next day, osmotic minipumps were implanted subcutaneously between the scapulae for continuous delivery of different treatments at a rate of 0.25 μl h⁻¹ over a time period of 14 days (ALZET microosmotic pump, Model 1002). Mice were anesthetized with ketamine (100 mg kg⁻¹) and xylazine (15 mg kg⁻¹) for the procedure. Treatments included un conjugated AON (20 μg per day × 14 days = 1.8 μmol kg⁻¹), αCD19–SON (20 μg per day × 14 days = 14 mg kg⁻¹ or 93.3 nmol kg⁻¹), control AON immuno conjugate αCD7–AON (20 μg per day × 14 days = 14 mg kg⁻¹ or 93.3 nmol kg⁻¹) and αCD19–AON (20 μg per day × 14 days = 14 mg kg⁻¹ or 93.3 nmol kg⁻¹). All SCID mice were electively killed at 120 day unless they died or became moribund earlier due to their disseminated leukemia. At the time of their death or killing, mice were necropsied to confirm leukemia-associated marked hepatomegaly and/or splenomegaly. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6-μm tissue sections were prepared and stained with hematoxylin–eosin. For the analysis of the SCID mouse xenograft data, event-free survival times were measured from the day of inoculation of leukemia cells to the day of death or killing. The probability of survival was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method, as previously reported. We used the Mantel–Cox log-rank test to assess the effects of various treatments on the probability of survival of SCID mice inoculated with LC1;19 cells, as reported. Statistical power and sample size analysis were performed at the 5% significance level and 95% power using a one-tailed, two-sample test for differences in proportions assuming normal approximations. For these experiments, 12 animals per group for pairwise comparisons provided sufficient power to detect significant differences at the 95% power level. With a total sample size of 75 (60 controls versus 15 CD19–AON mice), we can detect 23% increase in survival at 95% power.

Results

Up-regulated CD19 expression as a biomarker for t(1;19)− B-lineage ALL

CD19 is a B-lineage-specific surface receptor that is expressed on the surface of leukemia cells from 85% of patients with ALL. It is absent from parenchymal cells of nonhematopoietic organs, circulating blood myeloid and erythroid cells, T lymphocytes, and bone marrow stem cells. Gene expression profiling of primary leukemic cells from matched pair relapse vs. diagnosis bone marrow specimens of 59 patients with B-lineage ALL who relapsed showed similar expression levels for CD19 (Fig. 1, Table 1) (fold-difference relapse vs. diagnosis = 0.96, P = 0.68). Therefore, CD19 is uniquely suited to serve as a target for biotherapy against newly diagnosed as well as relapsed B-lineage ALL. Expression profiles for CD19 and CD22 transcripts were highly correlated forming a subcluster in the hierarchical cluster representation. Intriguingly, comparison of CD19 expression levels in primary leukemic cells in diagnostic specimens from patients who experienced an early (N = 40; time to relapse < 36 months) versus late relapse (N = 19; time to relapse ≥ 36 months) revealed a trend toward higher expression levels for early relapse cases (fold difference early vs. late relapse: 1.44, P = 0.06, Table 2), suggesting that CD19 may be a biomarker for subpopulations of patients who are at high risk for treatment failure and early relapse, such as those with t(1;19)/E2A–PBX1+ ALL. A similar borderline significant increase was detected for expression of the IL7R gene (Table 2).
Fig. 1  Surface receptor gene expression profiles of primary leukemic cells from matched pair relapse vs. diagnostic bone marrow specimens of ALL patients. 
Gene expression values for leukemic cells in matched pair specimens taken from 59 B-lineage ALL patients at diagnosis and then at relapse (combined from GSE3912, N = 32 and GSE18497, N = 27). RMA-normalized values for the GSE18497 dataset and the MA55-signal intensity values for the GSE3912 dataset were log_{10} transformed and mean centered to the average value for the diagnosis samples for each gene transcript in each study. A two-way agglomerative hierarchical clustering technique was used to organize expression patterns using the average distance lineage method such that genes (rows) having similar expression across patients and patients with similar gene expression profiles were grouped together (average distance metric).

Dendrograms were drawn to illustrate similar gene-expression profiles from joining pairs of closely related gene expression profiles, whereby genes joined by short branch lengths showed most similarity in expression profiles across patients and genes. The heat map represents the color-coded expression value for 59 matched pair diagnostic and relapse samples reported as mean centered expression value relative log_{10} transformed diagnosis samples.

Table 1  Surface receptor gene expression in ALL cells at initial diagnosis vs. subsequent first relapse

<table>
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<th>Gene symbol</th>
<th>Fold (relapse/diagnosis)</th>
<th>Paired t-test P-value</th>
<th>Cluster order</th>
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<tr>
<td>CD19</td>
<td>0.96</td>
<td>0.68</td>
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<tr>
<td>CD22</td>
<td>0.93</td>
<td>0.62</td>
<td>2</td>
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<tr>
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<td>0.50</td>
<td>3</td>
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<td>0.90</td>
<td>4</td>
</tr>
<tr>
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<td>1.04</td>
<td>0.96</td>
<td>5</td>
</tr>
<tr>
<td>IGF1R</td>
<td>1.12</td>
<td>0.89</td>
<td>6</td>
</tr>
<tr>
<td>CD40</td>
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</table>

Paired t-tests were performed for the combined log_{10} mean centered values from GSE3912 and GSE18497 matched pair datasets (N = 59 pairs). Fold increase in relapse and p-values are depicted.

We next set out to compare the CD19 expression levels of primary leukemic cells from 23 patients with t(1;19)/E2A–PBX1+ ALL vs. 184 patients with t(1;19)/E2A–PBX1+ ALL in a cohort of 207 patients with high risk B-lineage ALL (GSE11877). Notably, the gene for CD19 was expressed at significantly higher levels in the t(1;19)/E2A–PBX1+ subset (fold difference: 1.79, P = 0.00013; Fig. 2). Of 9 receptor genes examined, IL7R was the only other gene besides CD19 that was expressed at higher levels in t(1;19)/E2A–PBX1+ cells, but it is not a B-lineage specific receptor like CD19 and is strongly expressed in T-cells and T-cell precursors.27 In agreement with previous reports,13 the lymphoid stem cell marker CD34 was expressed at very low levels in t(1;19)/E2A–PBX1+ cells (fold difference: 0.20, P = 9.9 × 10^{-20}). Transcripts for CD40, IL2RA, and IL3RA were also significantly down-regulated in E2A–PBX1+ patients (Fig. 2).

Construction of an anti-CD19 immunol conjugate for targeting t(1;19)/E2A–PBX1+ ALL cells with an anti-sense oligonucleotide

A disulfide-linked immunol conjugate of a 5’-amino-modified 24 mer phosphorothioate anti-sense E2A–PBX1 oligonucleotide with a mAb specific for a CD19 receptor (αCD19-AON) was prepared as a CD19-directed and leukemia-specific anti-sense oligonucleotide (AON) targeting E2A–PBX1 fusion transcript positive B-lineage ALL cells with a t(1;19) chromosomal translocation (Fig. 3A–C). The CD19-specific immunoreactivity of this immunol conjugate with B-lineage ALL cells was confirmed by using standard ligand-binding assays23 using a ^{3}H-labeled radioactive preparation (specific activity: 0.085 μCi μg^{-1} ~ 6.6 × 10^6 cpm nmol^{-1}). At a 300 nM concentration of the αCD19–AON immunol conjugate, B-lineage ALL cells specifically bound 0.94 ± 0.38 × 10^5 molecules per cell. This specific binding was abolished by a 50-fold molar excess of unconjugated αCD19–mA, but not by a 50-fold molar excess of unconjugated control αCD7 mAb.

The destination of surface-bound αCD19–^{3}H-AON molecules was traced in B-lineage ALL cells (Fig. 3C). After a total of 23 h
treatment with the immunoconjugate, leukemia cells were washed to remove the unbound immunoconjugate, homogenized, and nuclei were separated by centrifugation at 600 g. Of 86 pmol of 3H-AON molecules associated with 135 × 10^6 cells (~3.8 × 10^5 molecules per cell), 82.6% (~3.1 × 10^5 molecules per cell) were found in the nuclear pellets. The various subcellular components contained in the supernatants were gated and separated by centrifugation at 600 g. Various cellular components were fractionated on Percoll density gradients. A substantial portion (Percoll gradient fractions 20–25, density range 1.05–1.06) (Fig. 3C). 24.2% of the cpm (4.2% of the total cell associated cpm or 0.2 × 10^5 molecules per cell) was localized in the plasma membrane (Percoll gradient fractions 3–6, density range: 1.05–1.06) (Fig. 3C). 24.2% of the cpm (4.2% of the total cpm or 0.16 × 10^5 molecules per cell) representing internalized aCD19–3H-AON was associated with the soluble cytoplasmic fraction (Percoll gradient fractions 1 and 2, density gradient <1.05). In contrast, only 2.3% of the cpm (0.4% of the total cpm or 0.15 × 10^5 molecules per cell) banded in the region near the bottom of the gradient where the lysosomes are located (Percoll gradient fractions 20–25; density range 1.063–1.08; Fig. 3C).

The ability of aCD19–AON to knock down the expression levels of E2A–PBX1 fusion transcripts in t(1;19)− B-lineage ALL cells with 1 µg ml^−1 (~6.7 nM) aCD19–AON for 96 h resulted in selective depletion of E2A–PBX1 fusion transcripts (Fig. 4A). In contrast, E2A–PBX1 expression levels were not affected by (a) SPDP-modified unconjugated aCD19–mAb (100 µg ml^−1 = 670 nM), (b) control aCD9 immunonjugate prepared with a scrambled oligonucleotide (aCD9–SON) (10 µg ml^−1 = 67 nM), or (c) control aCD7 immunonjugate prepared with the E2A–PBX1 AON (aCD7–AON) (10 µg ml^−1 = 67 nM), indicating that both the AON moiety and the targeting CD19-specific mAb moiety are required for the biological activity of aCD19–AON. Furthermore, preincubation of
levels, the ABL transcript levels were not affected by αCD19–AON after 96 h treatment at 67 nM concentration demonstrating the selective nature of the αCD19–AON-mediated knock-down of E2A–PBX1 transcript levels in LC1;19 cells (Fig. 4B).

In vitro and in vivo anti-leukemic activity of αCD19–AON against t(1;19)/E2A–PBX1+ ALL cells

We next used confocal microscopy to examine the cytotoxicity of αCD19–AON against E2A–PBX1+ B-lineage ALL cells. Treatment with 6.7 nM αCD19–AON caused apoptotic destruction of E2A–PBX1+ LC1;19 cells within 96 h, whereas SPDP-modified unconjugated αCD19–mAb (670 nM, αCD19–SON) (67 nM), or αCD7–AON (67 nM) did not affect their viability (Fig. 4C).

Likewise, αCD19–AON at picomolar to low nanomolar concentrations (but not αCD19–mAb, αCD19–SON, or αCD7–AON) killed clonogenic LC1;19 cells with >99% clonogenic death achieved at concentrations ≥ 6.7 nM (linear contrast, $R^2 = 0.95$, t-values > 12, P-value < 0.00001 for all control versus αCD19–AON treatments) (Table 3, Fig. 4D). Neither γ-rays nor VCR were effective in killing clonogenic LC1;19 cells. Clonogenic fractions of the E2A–PBX1+ B-lineage leukemia cell lines ALL1 (BCR-ABL+) or RS4;11 (MLL-AF4+) expressing the target surface receptor CD19 were not killed by αCD19–AON (linear contrast, P-value = 0.42 and 0.56 for ALL1 and RS4;11, respectively). Similarly, αCD19–AON did not inhibit the clonogenic growth of the CD19 t-lineage ALL cell line MOLT3 even at 67 nM (linear contrast, $P = 0.44$) (Fig. 4D, Table 3). MOLT-3 was moderately sensitive to both γ-rays and VCR (Table 3). These results demonstrate that this rationally designed immunoconjugate is selectively cytotoxic to E2A–PBX1+ B-lineage ALL cells expressing both CD19 which is recognized by its mAb moiety and E2A–PBX1 transcripts, which represent the leukemia-specific molecular target of its AON moiety.

We next evaluated the anti-leukemic efficacy of αCD19–AON against E2A–PBX1+ human B-lineage leukemia in an established SCID mouse xenograft model of human t(1;19)+ ALL in which the E2A–PBX1+ B-lineage ALL cell line LC1;19 causes disseminated and invariably fatal leukemia in SCID mice (Fig. 5). All untreated control mice (N = 65) challenged with an intravenous inoculum of $1 \times 10^6$ LC1;19 cells died of overt leukemia with a median event-free survival (EFS) of 37.0 ± 0.1 days. At the time of death, all mice had massive hepatosplenomegaly due to leukemic infiltration. Histopathologic studies showed leukemic infiltration of multiple organs, including bone marrow, brain, liver, spleen, ovaries, and lungs. Involvement of bone marrow ranged from moderate, multifocal to diffuse infiltration with replacement of normal tissue elements by sheets of closely packed leukemic blasts resulting in total or near total effacement of the normal tissue architecture. Involvement of the CNS consisted of infiltration by leukemic blasts in the leptomeninges, and mild multifocal parenchymal infiltration. Likewise, all control mice treated with (a) unconjugated AON (20 µg per day × 14 days = 1.8 µmol kg$^{-1}$) or αCD19–SON (20 µg per day × 14 days = 14 mg kg$^{-1}$ or 93.3 nmol kg$^{-1}$; N = 25; median EFS: 49.0 ± 0.4 days) or (b) control AON immunoconjugate αCD7–AON (20 µg per day × 14 days = 14 mg kg$^{-1}$ or 93.3 nmol kg$^{-1}$; N = 10; median

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**Legend for Fig. 4**

**A.** LC1;19 cells with a 3–30-fold excess of unconjugated αCD19–mAb effectively blocked the αCD19–AON-induced knock-down of E2A–PBX1 expression in a concentration-dependent fashion (Fig. 4A). Unlike the leukemia-specific E2A–PBX1 transcript

**B.** LC1;19 cells were treated for 96 h with αCD19–AON, αCD7–AON, or αCD19–SON, stained with a rabbit polyclonal anti-tubulin antibody (green fluorescence) and the DNA-specific dye Toto-3 (blue fluorescence), and examined by laser scanning confocal microscopy for apoptotic changes, as described in Materials and methods. Cells treated with αCD19–AON (depicted in C1) showed classic signs of advanced apoptosis, including total loss of the tubulin fiber network, destruction of cytoplasm with irregular contours and vacuolation along with nuclear fragmentation and micronuclei formation. Apoptotic bodies containing remnants of nuclear and cytoplasmic material were detected. In contrast, control cells treated with αCD19–SON (depicted in C2) or αCD7–AON (depicted in C3) showed normal tubular architecture and normal round blue nuclei with no signs of damage. (D) The effects of the indicated treatments on the clonogenic survival of human ALL cell lines were examined using in vitro colony assays as described in Materials and methods. % Clonogenic death was calculated using the formula: % clonogenic death = 100 – [100 × (mean no. colonies per 10$^4$ cells plated after test treatment)/mean no. colonies per 10$^4$ cells plated after vehicle control treatment)]. Significant effect sizes ($p<0.0001$ for all contrasts) were observed for t(1;19)+ LC1;19 cells comparing αCD19–AON treatment and controls.
The effects of the indicated treatments on the clonogenic survival of human ALL cell lines were examined using in vitro colony assays as described in Materials and methods. % Clonogenic death was calculated using the formula: % Clonogenic death = 100 - [100 × (mean no. colonies per 10^4 cells plated after test treatment)/(mean no. colonies per 10^4 cells plated after vehicle control treatment)]. The results are shown as the mean number of colonies in the individual replicate samples are shown in brackets. Experiments 1 and 2 are two independent experiments.

**Table 3** Anti-CD19–AON kills clonogenic t(1;19)^+ ALL cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LCI;19 CD19^+ B-lineage ALL</th>
<th>RS4;11 CD19^+ B-lineage ALL</th>
<th>ALL-1 CD19^+ B-lineage ALL</th>
<th>MOLT3 CD19^+ T-lineage ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E2A–PBX1^1</td>
<td>MLL-AF4^1</td>
<td>BCR-ABL^1</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment #1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>298.5 (283, 314)</td>
<td>122 (109, 133)</td>
<td>193 (187, 199)</td>
<td>139.5 (134, 145)</td>
</tr>
<tr>
<td>zCD19–AON 670 pM</td>
<td>3 (2, 4)</td>
<td>99.0</td>
<td>232 (219, 245)</td>
<td>140.5 (124, 157)</td>
</tr>
<tr>
<td>zCD19–AON 6.7 nM</td>
<td>0 (0, 0)</td>
<td>&gt;99.6</td>
<td>190 (183, 197)</td>
<td>27 (23, 31)</td>
</tr>
<tr>
<td>zCD19–SON 6.7 nM</td>
<td>0 (0, 0)</td>
<td>&gt;99.6</td>
<td>10.4</td>
<td>197.5 (196, 199)</td>
</tr>
<tr>
<td>zCD19–mAb 670 nM</td>
<td>321 (305, 337)</td>
<td>0</td>
<td>65 (54, 76)</td>
<td>34.3</td>
</tr>
<tr>
<td>zCD7–AON 67 nM</td>
<td>312 (298, 326)</td>
<td>0</td>
<td>5.4</td>
<td>32.4</td>
</tr>
<tr>
<td>4 Gy γ-rays</td>
<td>246.3 (242, 251)</td>
<td>17.4</td>
<td>6.8</td>
<td>65 (54, 76)</td>
</tr>
<tr>
<td>VCR 25 µg ml⁻¹</td>
<td>299.3 (279, 320)</td>
<td>0</td>
<td>281.5 (273, 290)</td>
<td>121 (97, 145)</td>
</tr>
<tr>
<td><strong>Experiment #2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>209 (197, 221)</td>
<td>177.5 (175, 180)</td>
<td>281.5 (273, 290)</td>
<td>121 (97, 145)</td>
</tr>
<tr>
<td>zCD19–AON 670 pM</td>
<td>8 (7, 9)</td>
<td>96.2</td>
<td>210 (200, 220)</td>
<td>113 (101, 125)</td>
</tr>
<tr>
<td>zCD19–AON 6.7 nM</td>
<td>0 (0, 0)</td>
<td>&gt;99.5</td>
<td>268 (259, 277)</td>
<td>136 (133, 139)</td>
</tr>
<tr>
<td>zCD19–SON 6.7 nM</td>
<td>0 (0, 0)</td>
<td>&gt;99.5</td>
<td>190 (188, 192)</td>
<td>27 (23, 31)</td>
</tr>
<tr>
<td>zCD19–mAb 670 nM</td>
<td>191 (163, 219)</td>
<td>8.6</td>
<td>136 (133, 139)</td>
<td>78.6</td>
</tr>
<tr>
<td>zCD7–AON 67 nM</td>
<td>238.3 (225, 252)</td>
<td>0</td>
<td>281.5 (273, 290)</td>
<td>32.4</td>
</tr>
<tr>
<td>4 Gy γ-rays</td>
<td>245.5 (218, 273)</td>
<td>0</td>
<td>315.5 (303, 328)</td>
<td>54 (49, 59)</td>
</tr>
<tr>
<td>VCR 25 µg ml⁻¹</td>
<td>269.5 (251, 288)</td>
<td>0</td>
<td>256.5 (242, 271)</td>
<td>15 (13, 17)</td>
</tr>
</tbody>
</table>

The EFS: 43.0 ± 0.5 (d) rapidly died of disseminated leukemia with <10% survival at 60 days (Fig. 5). In contrast, zCD19–AON (20 µg per day × 14 days = 14 mg kg⁻¹ or 93.3 nmol kg⁻¹) delivering 74 µg kg⁻¹ per day of the AON (total amount of AON delivered: 1.48 µg per day × 14 days = 1 mg kg⁻¹) more than doubled the median EFS to 82.0 ± 1.9 days. While only 3% of control mice were alive at 60 days and none were alive at 90 days, 87% and 47% of zCD19–AON-treated mice remained alive at 60 days and 90 days, respectively (P < 0.0001) (Fig. 5).

**Discussion**

Here we first report a novel, biomarker-guided biotherapy strategy for personalized treatment of t(1;19) ALL. Inspired by the results of our bioinformatics studies that provided the first evidence in the gene for the B-lineage specific surface receptor CD19 is constitutively overexpressed in t(1;19) B-lineage ALL, we prepared a rationally-designed, disulfide-linked immunoconjugate of a 5-amino-modified 24 mer phosphorothioate antisense E2A–PBX1 oligonucleotide (AON) with a mAb specific for a CD19 receptor (zCD19–AON) as a CD19-directed and leukemia-specific biotherapeutic agent against E2A–PBX1^+ B-lineage ALL. Treatment of E2A–PBX1^+ leukemia cells with low nanomolar concentrations of zCD19–AON resulted in selective depletion of E2A–PBX1 transcripts and caused apoptotic destruction and abrogation of clonogenic growth. At a dose of 93 nmol kg⁻¹, zCD19–AON more than doubled the leukemia-free survival time of SCID mice challenged with radiotherapy-resistant highly aggressive human E2A–PBX1^+ B-lineage leukemia cells. Both the AON moiety and the targeting CD19-specific mAb moiety were required for the *in vitro* as well as *in vivo* anti-leukemic activity of zCD19–AON. The observed *in vitro* and *in vivo* anti-leukemic potency of the zCD19–AON immunoconjugate provides the first preclinical proof-of-principle that t(1;19)^+ high risk B-lineage ALL can be treated with leukemia-specific biotherapeutic agents that knock-down E2A–PBX1 expression.

Our data indicate that the E2A–PBX1 transcripts are important for the survival of t(1;19)^+ B-lineage ALL cells and their *in vitro* as well as *in vivo* clonogenic subpopulations. AON acts by targeting specific mRNAs through heteroduplex formation inside the cell, thereby inducing RNase H activation, translational arrest, or alternative splicing. The observed *in vitro* and *in vivo* anti-leukemic potency of the zCD19–AON immunoconjugate provides preclinical proof-of-principle that t(1;19)^+ high risk B-lineage ALL can be treated with leukemia-specific biotherapeutic agents that knock down E2A–PBX1 expression.

CD19 is a 95 kDa B-lineage restricted receptor molecule that functions as a key regulator of transmembrane signals in both B-cells and B-cell precursors. CD19 antigen is acquired at a very early stage of B-cell ontogeny, prior to rearrangement of immunoglobulin genes and expression of other B-precursor antigens such as CD10 and CD22. CD19 antigen is abundantly expressed on the malignant cells from B-lineage leukemia and lymphoma patients, but it is absent on the parenchymal cells of life-maintaining non-hematopoietic organs, circulating blood myeloid and erythroid cells, T-cells as well as bone marrow stem cells. In B-lineage ALL, CD19 antigen is expressed on candidate leukemic stem cell populations with *in vivo* clonogenic, leukemia initiating and propagating properties in xenograft models using immunocompromised mice. The very
favorable B-lineage leukemia/lymphoma vs. normal tissue expression profile of CD19 and its association with stemness properties of leukemic B-cell precursor populations make it an attractive molecular target for biotherapy in relapsed ALL.  

Several hundred thousand CD19 molecules located on the surface of each B-lineage leukemia/lymphoma cell are rapidly internalized upon ligation with anti-CD19–mAb or immunoconjugates.  

CD19 has tyrosine-based internalization motifs in its cytoplasmic domain that were predicted to bind the clathrin adaptor AP-2.  

Ingle et al. reported that CD19-directed mAb and mAb-drug conjugates are internalized by dynamin-dependent, clathrin-mediated endocytosis and their cellular uptake as well as intracellular trafficking mimic the endocytic pathway of transferrin. Their studies demonstrated that anti-CD19–mAb are transported to late endosomes and lysosomes within 3 h. Clathrin inhibitor chlorpromazine has been shown to inhibit the uptake of both transferrin and anti-CD19–mAb. Detailed immunoelectron microscopy studies by Pulczynski et al. showed that upon binding of anti-CD19–mAb, CD19 antigen on the surface of pre-B ALL cells is internalized within 30 min via plasmalemmlar pits, transferred through the endosomal compartment within 2 h, delivered to multivesicular bodies/late endosomes and lysosomes after 2 h, and recycled. Notably, the internalized anti-CD19–mAb–CD19 complexes showed close physical association with/attachment to the endosomal membrane. This close association of internalized anti-CD19–mAb with the endosomal membrane likely facilitates the endosomal leakage and/or escape of chemical or biological substances attached to them into the cytosol and provides a cogent explanation for the documented potency of toxin conjugates as well as drug conjugates of anti-CD19–mAb against CD19 leukemia/lymphoma cells. The pharmacological effectiveness of oligonucleotide-based therapeutics depends on their cellular uptake, intracellular trafficking, endosomal release, and productive delivery to their target subcellular compartments. The observed potency of the anti-CD19–mAb–AON immunoconjugate against t(1;19) ALL cells demonstrates that this biotherapeutic agent is inherently capable of effective delivery of AON molecules to relevant subcellular compartments where they can target specific mRNAs through heteroduplex formation.

While our proof-of-concept study employed AON to knock down E2A–PBX1 transcript expression in leukemia cells, there are other technology platforms that could be used for the same purpose, including RNA interference (RNAi) using small interfering RNAs (siRNA) that can be delivered using nanoparticles. In recent years, several recombinant fusion proteins that consist of a cell surface targeting moiety (e.g. an antibody fragment or ligand) and an oligonucleotide complexation moiety (e.g. truncated protamine) have been designed for targeted delivery of siRNA. Alternatively, mAb mediated delivery of AON to target cancer cells may provide an effective means for selective knock down of cancer-specific oncogenic genes, such as the E2A–PBX1 fusion gene of t(1;19) ALL cells. The described anti-CD19–AON immunoconjugate and its derivatives may offer an effective treatment for t(1;19) B-lineage ALL. Whether AON immunoconjugates will prove superior to immunoconjugates containing cytotoxic drugs or immunomodulatory bifunctional anti-CD19 biotherapeutic agents should be examined in appropriate preclinical and clinical settings.

Conflicts of interest statement

The authors have no conflicts to disclose.
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