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Scientific Section: Lymphoid Neoplasia

Article Title: Parthenolide eliminates leukemia initiating cell populations and improves survival in xenografts of childhood acute lymphoblastic leukemia

Running Title: PTL eliminates LIC in childhood ALL

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Key Points
- First report demonstrating in vivo elimination of multiple LIC populations from childhood ALL cases using animal models.
- In vivo models of leukaemia are essential for drug evaluation studies.

Abstract
Around 20% of children with acute lymphoblastic leukemia (ALL) relapse due to failure to eradicate the disease. Current drug efficacy studies focus on reducing leukemia cell burden. However, if drugs have limited effects on leukemia-initiating cells (LIC), these cells may expand and eventually cause relapse. Parthenolide (PTL) has been shown to cause apoptosis of LIC in acute myeloid leukemia. Here, we assessed the effects of PTL on LIC populations in childhood ALL. Apoptosis assays demonstrated that PTL was effective against bulk B- and T-ALL cells, while the CD34+/CD19−, CD34+/CD7− and CD34− subpopulations were more resistant. However, functional analyses revealed that PTL treatment prevented engraftment of multiple LIC populations in NSG mice. PTL treatment of mice with established leukemias from low- and high-risk cases resulted in survival and restoration of normal murine hemopoiesis. In 3 cases only, disease progression was significantly slowed in mice engrafted with CD34+/CD19− or CD34+/CD7− and CD34− cells but not prevented, demonstrating that individual LIC populations within patients have different responses to therapy. These observations indicate that PTL may have therapeutic potential in childhood ALL and provide a basis for developing effective therapies that eradicate all LIC populations to prevent disease progression and reduce relapse.
**Introduction**

Childhood acute lymphoblastic leukemia (ALL) is a heterogeneous disease in terms of karyotype, immunophenotype and blast morphology.\(^1\)\(^-\)\(^3\) Several genome-wide analyses have described additional genetic changes within individual leukemia clones.\(^4\)\(^-\)\(^6\) Consequently, studies which increase our understanding of the biology and evolution of this disease should provide information on leukemogenic pathways for therapeutic targeting. Such studies may help us understand why initial therapies can induce remission but some cases then relapse,\(^7\)\(^-\)\(^9\) especially as many relapses occur in the low-risk groups.

ALL cells that can generate leukemias in immune deficient mice, termed leukemia initiating cells (LIC), were initially thought to be rare and have a hierarchical structure.\(^10\)\(^-\)\(^13\) Some of these LIC, particularly CD133\(^+\)/CD19\(^-\) cells, were resistant to treatment with dexamethasone and vincristine,\(^13\) commonly used in the induction phase of therapy for ALL, so they may survive therapy and eventually cause disease relapse. However, more recent studies using NOD/LtSz-scid IL-2R\(\gamma\)null (NSG) mice have revealed that both primitive and more differentiated ALL subpopulations can initiate and maintain acute leukemias in this strain.\(^5\)\(^,\)\(^14\)\(^-\)\(^18\) We and others have shown that CD34\(^+\)/CD19\(^+\), CD34\(^+\)/CD19\(^-\) and CD34\(^-\) B cell precursor (BCP) ALL subpopulations contain LIC.\(^15\)\(^,\)\(^18\) In childhood T-ALL we found that CD34\(^+\)/CD7\(^+\), CD34\(^+\)/CD7\(^-\) and CD34\(^-\) subpopulations had LIC properties.\(^18\) Another study described CD7\(^+\)/CD1a\(^-\) and CD7\(^+\)/CD1a\(^+\) LIC from 1 pediatric and 2 adult T-ALL cases\(^19\) and a study of mature/cortical T-ALL found LIC in both CD34\(^+\)/CD7\(^+\) and CD34\(^-\)/CD7\(^+\) populations.\(^20\) The in vivo repopulating capacity has been shown to vary depending on the immunophenotype\(^18\) and genotype of the LIC.\(^5\) These findings demonstrate that LIC are more abundant than earlier studies suggested and indicate that leukemia evolution may have a branching structure rather than a hierarchical one. This has significant implications for therapy of leukemias. Elimination of the population, which contains the greatest proportion of LIC, or has the greatest self-renewal potential may just lead to another LIC population evolving and expanding to maintain the disease. Consequently, it will be important to develop therapies that can eliminate all populations with LIC potential to prevent further evolution and recurrence. The challenge is to find a way to specifically target LIC without causing toxicity to normal cells.
Parthenolide (PTL) has been recently investigated as a potential chemotherapeutic agent for acute myeloid leukemia (AML) and chronic lymphocytic leukemia.\textsuperscript{21-26} PTL is a naturally occurring sesquiterpene lactone used in the treatment of fever, migraines, rheumatoid arthritis and as an anti-inflammatory agent.\textsuperscript{27-29} PTL can induce apoptosis through a range of responses, such as inhibition of nuclear factor molecule kappa B (NF-κB), p53 activation and increase of reactive oxygen species.\textsuperscript{21,23} PTL and DMAPT, an analog of PTL, have been shown to be effective in AML while sparing normal hemopoietic stem cells (HSC).\textsuperscript{21,22} PTL can induce apoptosis in primary B-ALL cells\textsuperscript{22} and cell lines.\textsuperscript{30,31} To date there are no reports on the effects of PTL on LIC populations in pediatric ALL. The aim of this study was to evaluate the effects of PTL on childhood ALL cells, especially subpopulations that have LIC activity, including those that have been shown to be resistant to current therapeutic agents.\textsuperscript{13}
Methods

Patient cells
Bone marrow (BM) cells from children (median age, 6 years 8 months; range, 6 months - 19 years) with BCP ALL and T-ALL at presentation or relapse were collected with approval of University Hospitals Bristol NHS Trust. Detailed characteristics of the patient samples are shown in Table 1. Samples were selected on the basis of availability of material for study only. Normal BM (NBM) and peripheral blood (PB) samples were obtained from consented healthy donors. Cells were separated using Ficoll-Hypaque (Sigma-Aldrich, Poole, UK), mononuclear cells (MNC) were suspended in Iscoves modified Dulbecco's medium (IMDM; Invitrogen, Paisley, UK) with 90% fetal calf serum (FCS; Invitrogen) and 10% dimethyl sulfoxide (DMSO; Manor Park Pharmaceuticals, Bristol, UK) and stored in liquid nitrogen. Mean viability of samples on thawing was 83±17% for ALL samples and 71±17% for normal samples.

Cell sorting
B-ALL cells were stained with antibodies against CD34 (clone 8G12) and CD19 (clone 4G7). T-ALL cells were stained with anti-CD34 and anti-CD7 (clone M-T701). Normal BM and PB samples were stained with anti-CD34 and anti-CD38 (clone HB7). IgG1 antibodies were used as isotype controls (all BD Biosciences, Oxford, UK). Cells were sorted using a Becton Dickinson Influx Cell sorter (BD Biosciences), using Spigot 6.1.9 software, on the basis of fluorescence intensity after gating on 7-aminoactinomycin D (7AAD; Sigma-Aldrich) negative cells with low forward and side scatter. Details of the proportions of nucleated cells in the sorted populations are provided in Supplemental Table 1. Sorting was performed using the maximum purity setting and the purity of sorted subfractions from each sample was checked during and after sorting.
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<th>Patient</th>
<th>Subtype</th>
<th>Karyotype</th>
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<th>Sex</th>
<th>Disease status at biopsy</th>
<th>MRD† Risk Status</th>
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<td>28</td>
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<td>46XY</td>
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<td>M</td>
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<td>Diagnosis</td>
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<tr>
<td>34</td>
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<td>46XY</td>
<td>13</td>
<td>M</td>
<td>Diagnosis</td>
<td>High</td>
</tr>
</tbody>
</table>

*ALL secondary to Downs syndrome; † MRD risk status at day 28; IM, intermediate; ND, not determined.
In vivo studies

NSG mice were bred and maintained at the University of Bristol Animal Service Unit. Mice were not preconditioned prior to inoculation. Cells were resuspended in 0.3mL IMDM + 5% HAS and injected into the lateral tail veins of 6-8 week old mice. Unsorted cells and sorted cell populations were inoculated at a range of doses to obtain estimates of LIC frequency in each subpopulation.

In ex vivo drug sensitivity assays, unsorted cells and sorted subpopulations, from ALL patients and normal donors, were treated with either PTL for 20 to 24 hours or DMSO + phosphate buffered saline (PBS; Invitrogen), as controls, prior to inoculation.

In survival assays, mice were inoculated with bulk ALL cells and sorted subpopulations. Mice were monitored weekly for the presence of human cells in PB aspirates. Once the level of human cells was ≥5%, animals were given daily intravenous doses of PTL (40mg/kg) or placebo (DMSO+PBS) for 9 days.22,32

In all assays, animals were monitored weekly for the presence of human cells, maintained for up to 20 weeks and killed electively or until they began to exhibit clinical symptoms of disease. The gross anatomy was inspected and femoral BM samples were removed for flow cytometric and cytogenetic/histologic analyses. Immunophenotypes of xenografts were examined using antibodies against human CD3, CD10, CD19, CD7, CD33, CD34, CD45 and murine CD45 (all BD Biosciences). Cytogenetic analysis by FISH was performed by Bristol Genetics Laboratory, Southmead Hospital, Bristol.

Cells harvested from BM of some engrafted mice were used for serial transplantation experiments. For comparison with primary transplants, equal numbers of human CD45 cells were inoculated into serial recipients. These cells were not enriched for any particular phenotype before evaluation in sequential xenografts.

In vitro drug sensitivity

Unsorted ALL cells and sorted subpopulations were co-cultured with increasing
concentrations of PTL (Sigma-Aldrich) for 20 to 24 hours in RPMI-1640 (Sigma-Aldrich) containing 10% FCS and 1% L-glutamine (Invitrogen). Unsorted and (HSC)-enriched (CD34+/CD38-) NBM samples were also co-cultured with the drug. Apoptosis and viability were assessed by flow cytometry using annexin V-FITC and propidium iodide (arcus biologicals, Modena, Italy).

**Western blotting and confocal microscopy**

Cells for Western blotting were prepared by the method described by Foka et al.\textsuperscript{33} Full details are provided in the Supplemental data.

**Statistical analysis**

LIC frequencies were determined by Poisson statistics using L-Calc software (StemCell Technologies Inc). Analysis of variance, followed by Tukey’s post-hoc testing, was used to compare drug responses between ≥3 populations. Matched paired T-tests were used to compare viability and engraftment levels between untreated and drug-treated groups. T-tests assuming unequal variance were used to compare drug-treated ALL populations with treated HSC. Data from survival assays were analyzed using the log-rank test of survival distribution after treatment.
Results

LIC frequency in ALL subpopulations

Cells from 15 cases were sorted based on expression of CD34 and CD19 (B-ALL cases, Fig. 1A) and CD34 and CD7 (T-ALL cases, Fig. 1B). Cells were inoculated at a range of doses to give an estimate of the frequency of LIC (Table 2). LIC frequency in sorted subpopulations varied from 1 in 700 to 1 in 9x10^5 but was highest in CD34+/CD19- cells in B-ALL cases and CD34+/CD7- cells in T-ALL cases. As the number of sorted cells available limited the extent of the dilution analysis, these results are likely to be an underestimate of LIC frequencies. Since LIC were readily detectable in all sorted subfractions, we subsequently assessed the effects on PTL on these subpopulations.

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<tr>
<th>Patient ID</th>
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<td>1 / 3x10^5</td>
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<td>Cell Dose</td>
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<td>4x10^3 - 5x10^6</td>
<td>2x10^2 - 5x10^5</td>
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Viability of PTL-treated ALL cells

PTL was initially tested in vitro at a dose range from 0.5-10µM (Fig. 2A) in 10 cases. The IC\(_{50}\) was reached at 7.5µM in 9 cases but 10µM PTL reduced the viability of all cases to ≤43.3%. This higher dose had no effect on the viability of normal HSC (p=0.91, Fig. 2B). Therefore, 10µM PTL was used routinely thereafter to maximize effects in all patients.

Unsorted cells from 16 of 23 B-ALL cases responded to the drug (<38% viable), 4 showed a partial response (58-71%) and 3 were largely unaffected (>87% viable). There was a significant reduction in viability in all populations following treatment, compared to untreated controls (P≤0.043). When individual subpopulations were compared, CD34\(^+\)/CD19\(^-\) cells were least affected with 81.3±36% surviving (P=0.0005, F=6.64, F\(_{crit}\)=2.71). Less than 42±40% cells from unsorted samples or other subpopulations remained viable after treatment. Individual results are shown in Supplemental Table 2.

When B-ALL cases were grouped according to MRD risk status, where available, into low (n=12) and high risk (n=8), a similar response pattern was observed in the low risk group (Fig 3A), with CD34\(^+\)/CD19\(^-\) cells most resistant (87.6±26% viable, P=0.0001, F=11.31, F\(_{crit}\)=2.82). The viability of the other subpopulations was ≤35±33%, significantly lower than HSC (100±14% viable, p≤0.0004). In the high-risk group, there was no significant difference in viability between the sorted subpopulations (P=0.53, F=0.75, F\(_{crit}\)=2.90). High-risk cases were less responsive and CD34\(^+\)/CD19\(^-\) and CD34\(^-\) subpopulations were most resistant (71.3±45% and 70.3±51% viable, respectively). These responses were not significantly different from results obtained with HSC (0.12<P<0.43). However, the viability of treated unsorted and CD34\(^+\)/CD19\(^+\) cells was significantly lower than treated HSC (P≤0.013).

The effect of PTL was also investigated in B-ALL subpopulations using CD133 (Fig. 3C, Supplemental Table 3). CD133\(^+\)/CD19\(^+\) and CD133\(^+\)/CD19\(^-\) subpopulations from 2 patients used above (pts 8, 15) and 4 additional cases (pts 4, 5, 9, 18) were sorted and treated with 10µM PTL. In this cohort, a greater response to PTL was observed using unsorted cells (13.7±17% viable) compared to the larger cohort used above
and only 5.9±5% CD133+/CD19+ cells survived. PTL had a reduced effect on the CD133+/CD19− subpopulation (71.6±21% viable) compared to the other populations assayed (P=0.0002, F=20.91, F_{crit}=4.10), but this was significantly reduced compared to results from HSC (P=0.032).

Seven of 9 T-ALL cases were affected by PTL (<36% viable) and a partial response was observed in the 2 remaining cases (62-69% viable, Fig. 3D). Only 27.2±27% unsorted T-ALL cells survived treatment while 48.4±29% CD34+/CD7+ cells, 54.9±41% CD34+/CD7− cells and 57.8±74% CD34− cells remained viable (P=0.53, F=0.76, F_{crit}=3.01). When treated populations were compared with their untreated counterparts there was a significant reduction in the viability in all populations (P≤0.039), except CD34− cells (P=0.105). This less marked effect of PTL on CD34− cells was largely attributable to 2 cases where these cells were unaffected by PTL, while in the remaining cases <16% CD34− cells survived (P=0.04 cf. untreated). The proportions of unsorted, CD34+/CD7+ and CD34+/CD7− T-ALL cells surviving PTL treatment were significantly reduced compared to HSC (P≤0.018).

**In vivo engrafting capacity of PTL-treated cells**

Unsorted cells and sorted subpopulations from 5 B-ALL cases and 5 T-ALL cases were treated with 10μM PTL for 20-24 hours prior to inoculation to assess whether PTL had any effects on the ability of cells to engraft NSG mice (Fig. 4A). Groups of 2-4 mice were inoculated with each population assayed. Cell doses which had previously resulted in good engraftment levels were used. The number of sorted cells inoculated varied due to phenotypically primitive cells representing only small proportions of the total leukemia blast population (Supplemental Table 1) and different immunophenotypes amongst patients. Treatment of unsorted, CD34+/CD19+ and CD34− B-ALL cells with PTL prior to inoculation completely prevented engraftment in every case (Fig. 4B). PTL treatment of CD34+/CD19− cells significantly reduced or prevented engraftment in 3 cases (pts 17, 22 & 28). In the other 2 cases, the engrafting capacity of CD34+/CD19− cells from patient 20 was unaffected and engraftment was higher in mice inoculated with PTL-treated cells from patient 6 (98±3%) than with untreated cells (76±12%, Supplemental Fig. 1). FISH analyses on cells recovered from mice engrafted with unsorted and all sorted subpopulations, confirmed these cells had the same karyotype as the patients at
diagnosis (91-100% FISH+). Likewise, cells recovered from animals engrafted with PTL-treated CD34+/CD19- cells from pts 6 and 20 carried the patient specific aberrations (95-100% FISH+).

Treatment with PTL completely prevented engraftment of unsorted T-ALL cells in 4 of 5 cases (Fig. 4C). In the remaining case (pt 32) engraftment was significantly reduced to 22±6% compared to untreated cells (77±9%, P<0.001). No engraftment was observed using PTL-treated CD34+/CD7+ and CD34- cells. In 3 cases PTL prevented engraftment of CD34+/CD7- cells and significantly reduced engraftment in 2 cases, from 98±3% to 15±4% (pt 30) and from 52±7% to 16±4% (pt 32), P<0.001. FISH analyses confirmed the engrafted cells had an aberrant karyotype (67-100% FISH+). PTL treatment of normal CD34+/CD38- HSC prior to inoculation did not have any detrimental effects on the functional capacity of these cells. Engraftment levels of treated cells were 120% relative to the untreated controls (P=0.25, Supplemental Fig. 2).

**Self-renewal capacity of resistant cells**

In the 3 cases where in vitro PTL treatment of LIC had little (CD34+/CD19-, pts 6, 20) or limited effects (CD34+/CD7-, pt 32) on engrafting capacity, cells recovered from murine BM were transplanted into secondary animals to assess the self-renewal ability of the PTL-treated cells (Supplemental Table 4). Cells were not re-sorted prior to inoculation into secondary recipients but they received equivalent numbers of human cells as their primary counterparts. Comparable levels of engraftment were observed in the primary and secondary recipients. Inoculated LIC differentiated in vivo to give rise to leukemias that were typical of the patient samples at biopsy. However, a proportion of the grafts from animals inoculated with PTL-treated or untreated CD34+/CD19- cells remained CD34+/CD19- (3.7±0.6%). Likewise, the majority of cells recovered from mice inoculated with treated or untreated CD34+/CD7- cells were CD34+/CD7- but 14.2±2.3% remained CD34+/CD7-, indicating self-renewal of these primitive LIC.
In vivo activity of PTL in NSG

Unsorted and sorted cell populations from 9 cases were inoculated into NSG and PTL was administered when leukemia was established in the mice (Fig 5A). Disease progression was prevented in PTL-treated animals, while the leukemia burden in placebo-treated animals continued to rise until they had all succumbed to disease within 9 weeks after treatment commenced. PTL significantly increased survival for each treated xenograft (P<0.0014, Fig. 5B & C). The median survival time of untreated mice was 29 days (range 2-60) after treatment commenced for those engrafted with sorted and unsorted B-ALL cells and 25 days (range 6-36) for T-ALL xenografts. Disease progression continued in only 4 PTL-treated xenografts: a CD34+/CD19- xenograft of pt 6 survived for 24 days after treatment commenced, untreated counterparts survived only 2 days. Treated CD34+/CD7- and CD34- xenografts from pt 32 survived 48 days and 46 days, respectively, while the untreated animals survived 25 and 6 days, respectively. One CD34- xenograft from pt 30 survived for 34 days; however, the untreated equivalents succumbed at day 17. Xenografts of pt 30 had high levels of leukemia prior to treatment (24.2-65.8%). Following treatment, the levels of human cells detected in treated mice decreased, with the exception of 1 CD34- xenograft noted above, and levels of murine hemopoietic cells increased (Supplemental Fig. 3). The leukemia burden continued to increase in untreated animals and they all had to be killed between 2-36 days from treatment commencing. All other treated xenografts were disease-free when electively killed up to 18 weeks from commencement of treatment. PTL significantly decreased the leukemia cell burden in all treated animals (P<0.002, Fig. 5D & E). With the exception of the 4 xenografts noted above, the majority of cells recovered from PTL-treated animals expressed murine CD45. A representative example of immunophenotypic analyses of BM cells from untreated and PTL-treated xenografts established with CD34+/CD19- cells from pt 20 is shown in Fig. 5F.
Confirmation of activated NF-κB in ALL

Since PTL is a potent inhibitor of NF-κB, we investigated whether this mechanism of action was operational in a cohort of samples, including cases where PTL treatment had limited effects. The effects of the drug on the expression of active and total p65, phospho-IκB and IκB in a representative sample are shown in Supplemental Fig. 4. In all cases, there was constitutive expression of active p65 and phospho-IκB. Following PTL treatment, expression of both molecules was reduced, demonstrating inhibition of NF-κB. Constitutive expression of NF-κB was also confirmed in unsorted and sorted subpopulations by confocal microscopy. Treatment of unsorted samples resulted in inhibition of NF-κB. However, there was no difference in the levels of NF-κB expression in CD34+/CD19- cells or CD34+/CD7- and CD34- cells from the resistant cases. A representative example (pt 32) is shown in Supplemental Fig. 5.
Discussion
Outcomes for pediatric leukemias are increasingly successful. Nevertheless, 15-20% of patients relapse due to failure to eradicate the disease and most of these patients will not survive. Most drug efficacy studies focus on initial short-term effects, such as reducing the overall leukemia cell burden. However, if drugs have no effect on the LIC they may expand and eventually cause relapse. In this study we determined the phenotype of LIC *a priori* in a cohort of childhood ALL cases from mixed prognostic subgroups. LIC were detected in all sorted subpopulations assessed, in agreement with previous reports\(^{15,18-20}\) and were most enriched in the CD34\(^+\)/CD19\(^-\) and C34\(^+\)/CD7\(^-\) subpopulations in B- and T-ALL cases, respectively. Consequently, we assessed the effects of PTL on unsorted cells and on all subpopulations with LIC activity.

Apoptosis assays indicated that overall PTL reduced the viability of unsorted B-ALL cells to <38% and T-ALL cells to <28%. Patients who were classified as high risk by MRD were less responsive to PTL but an IC\(_{50}\) was reached at 10\(\mu\)M. At this dose PTL had no effect on the viability of CD34\(^+\)/CD38\(^-\) HSC. These findings are in agreement with Guzman et al, who reported that 53.5% unsorted B-ALL cells survived treatment with PTL.\(^{22}\) However, the effects of in vitro PTL treatment on CD34\(^+\)/CD19\(^-\), CD133\(^+\)/CD19\(^-\), CD34\(^+\)/CD7\(^-\) and CD34\(^-\) subpopulations were more limited and disappointing compared to the reported effects of PTL and DMAPT in AML.\(^{21,22}\) Nevertheless, a limitation of short-term assays is that they only provide a snapshot of the value of a drug at a specific time point. It is crucial to undertake functional studies of drug-treated cells to assess efficacy and targeting of specific cell populations.

When we assessed the capacity of in vitro PTL-treated subpopulations to repopulate NSG mice, engraftment of unsorted and most sorted cell populations was completely prevented or significantly reduced. Interestingly, engraftment of the CD34\(^+\)/CD19\(^-\), CD34\(^+\)/CD7\(^-\) and CD34\(^-\) subpopulations was severely impaired or completely prevented following PTL treatment, demonstrating that PTL can induce apoptosis in phenotypically primitive and more differentiated LIC and prevent disease establishment in vivo. Since we have previously shown that the functional ability of primitive LIC populations from patients 6, 13, 14, 16, 17 were unaffected by in vitro
treatment with dexamethasone and vincristine, the observed reduction in engrafting capacity of multiple LIC subpopulations, following PTL treatment, suggests this drug may be more effective. In vitro treatment of CD34+/CD19- cells from 2 B-ALL cases and CD34+/CD7- cells from 1 T-ALL case had only limited or no detrimental effects on the ability of these cells to engraft NSG mice. These cells could self-renew to repopulate serial NSG and recapitulate the original leukemia phenotype. Our results on the NSG engrafting capacity of in vitro PTL-treated cells are in agreement with results in AML. These findings demonstrate that PTL may have more potential as a therapeutic agent than the results from our short-term apoptosis assays indicated.

To further evaluate therapeutic potential we assessed the activity of PTL in vivo by treating animals with established leukemias. These models more closely mimic the clinical setting and disease progression since the leukemia cells disseminate to extramedullary organs once the BM is engrafted. PTL was well tolerated, with no toxicity observed and resulted in significant reductions in leukemia burden in the BM and extramedullary organs of mice engrafted with unsorted cells and sorted subpopulations. The overall outcome was survival of treated animals with restoration of murine hemopoiesis. Even animals with high leukemia cell burdens (>50% in PB) prior to treatment were successfully treated and survived until electively killed. Administration of PTL to xenografts established with CD34+/CD19- cells from pt 20 resulted in elimination of the leukemia and full recovery of murine hemopoiesis. In contrast, in vitro treatment of CD34+/CD19- LIC from this patient prior to inoculation into NSG had no effect on the engrafting capacity. These data highlight the importance of conducting drug testing in vivo using xenograft models.

Despite the clear and potent effects of in vivo PTL treatment, specific xenografts from 3 pts (6, 30, 32) were less responsive to the drug. Treatment of these xenografts significantly prolonged survival and reduced leukemia burden but did not prevent progression. Two were high-risk cases, 2 had relapsed at the time of sampling and only 1 is alive at present. PTL was effective in targeting LIC in other high-risk cases and those in relapse, so the lack of response observed in some xenografts cannot be attributed to these factors alone. The survival of all other treated animals and lack of
detectable human hemopoiesis, over 120 days after cessation of treatment, is a clear indication of the therapeutic potential of PTL.

Taken together these data indicate that PTL is effective against bulk leukemia and all LIC populations in the majority of cases studied. Had we only assessed the effect of PTL in vivo on unsorted leukemias, disease progression would have been prevented in every case. However, we have shown that 3 specific subpopulations, with both primitive and differentiated phenotypes, from 3 cases were resistant to this drug. Furthermore, where possible, we demonstrated PTL did not inhibit the NF-κB pathway, one reported mechanism of action, in the resistant LIC in these cases, which may be one reason for the observed lack of effect. Nevertheless, NF-κB was constitutively active in all unsorted samples analyzed. This concurs with a report that 39 of 42 heterogeneous pediatric ALL samples contained activated NF-κB complexes. A comprehensive mechanistic investigation of PTL was beyond the scope of this study. It is possible that the 3 resistant cases had more aggressive disease and that more intensive therapy would be required to ablate specific LIC in these patients.

ALL is currently being treated by a range of drugs, such as glucocorticoids, anthracyclines, vinca alkaloids, asparaginase, alkylating agents and antimetabolites. Despite their different modes and sites of action, most of those agents have been shown to induce NF-κB activation which, ironically, is a self-protective mechanism for the cell. Using NF-κB inhibitors in combination with chemotherapeutic agents would potentially improve clearance of leukemia cells. The effects of PTL on AML cells can be enhanced when it is used in combination with agents such as with mTOR and PI3K inhibitors. Bortezomib, an inhibitor of the ubiquitin proteasome pathway has been shown to be active in combination with several agents and act synergistically with the mTOR inhibitor RAD001 to kill ALL cells in vitro. Therefore, by combining PTL or DMAPT with mTOR/proteasome inhibitors it may be possible to achieve greater toxicity to pediatric ALL cells. It will be of interest to investigate such drug combinations in the resistant cases from our cohort.

This study represents the first report demonstrating in vivo ablation of multiple LIC
populations in childhood ALL. PTL was not dependent on expression of specific cell surface markers and was not restricted to specific subtypes. The findings add to the evidence that some LIC remain resistant to available agents and these cells could subsequently cause relapse. This report also highlights the importance of using animal models of leukemia to conduct drug evaluation studies, rather than just short-term viability analyses.
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Authorship
P.D. processed samples, designed and performed experiments and wrote the report.
C.V.C. processed samples, performed experiments and commented on the report.
J.P.M. facilitated sample collection, collated the clinical data information and commented on the report.
A.B. conceived and designed the study, performed in vivo experiments and wrote the report.

Conflicts of Interest
The authors have no competing financial interests to declare.
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Figure Legends

Figure 1  Assessment of LIC content of sorted ALL subpopulations
B-ALL cells from 8 patients (2, 6, 16, 17, 20, 22, 23, 28) were sorted for expression of CD34 and CD19 (A) and cells from 7 T-ALL cases (29-32, 34-36) were sorted for expression of CD34 and CD7 (B). Unsorted cells and sorted subpopulations were inoculated into NSG mice to evaluate the LIC content. Two to four mice were injected per cell dose. Each patient is represented by a specific symbol and each symbol depicts the leukemia engraftment detected in the BM of an individual mouse.

Figure 2  Response of ALL cells and normal HSC to PTL
(A) Percentage of viable ALL cells after PTL treatment in 7 B-ALL cases (open symbols) and 3 T-ALL cases (closed symbols). PTL was used at a dose range from 0.5 to 10µM and viability was assessed at 20 to 24 hours by flow cytometry using Annexin V and PI. Samples depicted with dashed lines are high MRD risk cases. (B) Viability of CD34+/CD38- HSC from normal BM treated with PTL (10µM) compared to untreated controls (n=6).

Figure 3  Viability of B- and T-ALL populations after PTL exposure
(A) Low MRD risk (n=12) and (B) high MRD risk (n=8) B-ALL subpopulations were sorted using antibodies against CD34 and CD19. (C) Six cases were sorted using antibodies against CD133 and CD19. Squares represent the low risk cases, triangles intermediate risk and circles high-risk cases. (D) Subpopulations from nine T-ALL cases were sorted using antibodies against CD34 and CD7. Two of the 9 cases were low MRD risk and are depicted with open squares. High MRD risk cases are depicted with filled circles. Graphs show the proportion of viable B- and T-ALL subpopulations following exposure to 10µM PTL. Data is expressed as a percentage of untreated controls, each symbol represents results from individual patients, horizontal bars represent mean viability. ** P ≤ 0.01, *** P≤0.001.

Figure 4  In vivo propagating ability of PTL-treated ALL subpopulations
(A) Unsorted and sorted ALL cells were treated with PTL for 20-24 hours in vitro then subsequently injected into NSG mice. Cells were inoculated at the following doses per mouse:- unsorted (1-5x10^6), CD34+/CD19+ (8x10^4-1x10^6), CD34+/CD19-
(1.8x10^3-1.2x10^5), CD34^- (1.5-2x10^6 B-ALL), CD34+/CD7^- (1-1.5x10^6), CD34+/CD7^- (4-6x10^4) and CD34^- (1.5-5x10^6 T-ALL). After 4-12 weeks, leukemia cell engraftment in BM was analyzed by flow cytometry using a panel of antibodies. (B and C) The levels of engraftment attained using unsorted and sorted subpopulations from 5 B-ALL cases (B) and from 5 T-ALL cases (C). Each symbol depicts the engraftment level of human leukemia cells, from the patients indicated, measured in BM of individual mice. Closed symbols represent mice inoculated with untreated cells, opens symbols represent mice inoculated with PTL-treated cells. Solid horizontal bars represent mean engraftment levels using untreated cells. Dashed bars represent mean engraftment levels using PTL-treated cells.

**Figure 5 PTL improves the survival of NSG mice engrafted with ALL subpopulations**

(A) NSG mice were transplanted with bulk and sorted subpopulations from B-ALL and T-ALL cases. Once engrafted, mice were treated for 9 days with PTL (40 mg/kg/day) and monitored thereafter. (B &C) Kaplan-Meier plots of the survival of animals engrafted with B-ALL (B, pts 6, 20, 22, 27, 28) and T-ALL subpopulations (C, pts 30, 32, 34, 35). Time indicated is time from commencement of treatment, day 0. The numbers in parentheses signify the numbers of mice used in each group. (D & E) PTL decreases leukemia cell burden in xenografts from B-ALL populations (D) and T-ALL populations (E). The percentage of leukemia cells in the BM of individual animals from each inoculated population is shown. Each symbol represents a specific patient. ** P ≤ 0.01, *** P ≤ 0.001, compared to untreated animals. (F) Flow cytometric analysis of BM from mice engrafted with CD34+/CD19^- cells from pt 20. Results from an untreated and a treated mouse are shown. In histograms, grey peaks represent murine CD45, black peaks are isotype controls. Spleens removed from the treated mouse (left, PTL) and untreated mouse (right, U).
Figure 1  Assessment of LIC content of sorted ALL subpopulations

% Engraftment

Unsorted  CD34+/CD19+  CD34+/CD19-  CD34-

Unsorted  CD34+/CD7+  CD34+/CD7-  CD34-
Figure 2  Response of ALL cells and normal HSC to PTL

A

B

Viable (as % of untreated)

PTL (μM)

Viable Cells (%)

Untreated  10 μM PTL
Figure 3  Viability of B- and T-ALL populations after PTL exposure

(A) Low MRD Risk

(B) High MRD Risk

(C)

(D)
In vivo propagating ability of PTL-treated ALL subpopulations

A

Bulk and sorted ALL and normal cells + PTL 20-24h Transplant into NSG mice 4-12 weeks Harvest BM, spleen Analyses of engraftment

B

% Engraftment

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6 17 20 22 28

Unsorted CD34+CD19- CD34+CD19- CD34+CD19- CD34-

C

% Engraftment

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30 32 34 35 36

Unsorted CD34+CD7- CD34+CD7- CD34+CD7- CD34-
Figure 5  PTL improves the survival of NSG mice engrafted with ALL subpopulations

A) Weekly PB sampling

PTL daily for 9 days

Weekly PB sampling

Survival studies (B,C)

Analyses of engraftment (D-F)

B) Unsorted

(placebo n=10, PTL n=12)

\[ P=0.0013 \]

\[ 0.0 \text{ to } 1.0 \]

\[ 0 \text{ to } 100 \]

C) CD34+CD19+

(placebo n=9, PTL n=12)

\[ P=0.0001 \]

\[ 0.0 \text{ to } 1.0 \]

\[ 0 \text{ to } 100 \]

D) CD34+CD7+

(placebo n=10, PTL n=12)

\[ P=0.0005 \]

\[ 0.0 \text{ to } 1.0 \]

\[ 0 \text{ to } 100 \]

E) CD34+

\[ P=0.0001 \]

\[ 0.0 \text{ to } 1.0 \]

\[ 0 \text{ to } 100 \]

F) PTL-treated

\[ \text{huCD45-FITC} \]

\[ \text{Murine CD45-FITC} \]

\[ \text{huCD19-PE} \]

\[ \text{huCD45-PE} \]

\[ \text{huCD19-FITC} \]

\[ \text{huCD45-FITC} \]

\[ \text{Murine CD45-FITC} \]

\[ \text{PTL} \]

\[ U \]