Acute Sensitivity of Ph-like Acute Lymphoblastic Leukemia to the SMAC-Mimetic Birinapant


Abstract

Ph-like acute lymphoblastic leukemia (ALL) is a genetically defined high-risk ALL subtype with a generally poor prognosis. In this study, we evaluated the efficacy of birinapant, a small-molecule mimic of the apoptotic regulator SMAC, against a diverse set of ALL subtypes. Birinapant exhibited potent and selective cytotoxicity against B-cell precursor ALL (BCP-ALL) cells that were cultured ex vivo or in vivo as patient-derived tumor xenografts (PDX). Cytotoxicity was consistently most acute in Ph-like BCP-ALL. Unbiased gene expression analysis of BCP-ALL PDX specimens identified a 68-gene signature associated with birinapant sensitivity, including an enrichment for genes involved in inflammatory response, hematopoiesis, and cell death pathways. All Ph-like PDXs analyzed clustered within this 68-gene classifier. Mechanistically, birinapant sensitivity was associated with expression of TNF receptor TNFR1 and was abrogated by interfering with the TNFα/TNFR1 interaction. In combination therapy, birinapant enhanced the in vivo efficacy of an induction-type regimen of vincristine, dexamethasone, and l-asparaginase against Ph-like ALL xenografts, offering a preclinical rationale to further evaluate this SMAC mimetic for BCP-ALL treatment.

Introduction

Apoptosis plays a critical role in the development and homeostasis of multicellular organisms, as well as normal and malignant hematopoiesis (1, 2). The intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways are tightly regulated by pro- and anti-apoptotic proteins (3, 4). The inhibitor of apoptosis (IAP) family are small antiapoptotic proteins that promote cellular survival mediated by the TNF superfamily of surface receptors and actively prevent the induction of apoptosis by inhibiting caspase activation. To date, 8 IAPs have been identified in humans with X chromosome linked IAP (XIAP), cellular IAP1 (cIAP1) and cIAP2 playing the most prominent role in cell survival (5). SMAC (second mitochondria-derived activator of caspases), is an endogenous antagonist of IAPs and is released from the mitochondria in response to apoptotic stimuli, thus activating downstream caspases (6).

Several approaches have been utilized to target IAPs, and the development of various small-molecule inhibitors, including SMAC mimetics, has recently been reported (5, 7). Birinapant is a bivalent SMAC mimetic with high affinity for cIAP1/2 (8). Upon binding, it promotes autoubiquitination of cIAP1/2 and proteosomal degradation, leading to inactivation of NFKB survival signaling and TNFα-dependent apoptosis (8). In response to TNFα receptor ligation (primarily via TNF receptor type 1, TNFR1, CD120a), birinapant also promotes caspase-8/ RIPK1 (receptor interacting serine/threonine protein kinase-1) complex formation, resulting in activation of caspase-8 and subsequent apoptosis. In some situations whereby caspase-8 function is impaired, an alternative cellular death pathway can be activated, whereby RIPK1 promotes RIPK3 phosphorylation and subsequent necroptosis (9, 10). Birinapant is currently in clinical trials for both solid tumors and hematologic malignancies and has so far been well tolerated and demonstrated excellent pharmacokinetic properties (8).

Various antiapoptotic proteins including IAPs are overexpressed in many cancers (4, 11, 12) and are often associated with chemoresistance and treatment failure, making them an attractive target for therapeutic intervention (13). In pediatric acute myeloid leukemia, cIAP1 was reported to be highly expressed in comparison with normal tissues (14), and aberrant expression of XIAP correlated with poor prognosis in acute lymphoblastic leukemia (ALL; ref. 15). ALL accounts for approximately 80% of all pediatric leukemias (16) and, while the overall cure rate for pediatric ALL now exceeds 80%, certain high-risk subtypes still experience shorter remissions and lower survival rates. A high-risk subtype...
of B-cell precursor (BCP) ALL, termed Philadelphia chromosome–like (Ph-like) ALL, was identified on the basis of a gene expression classifier similar to BCR-ABL1–positive cases despite the absence of the BCR-ABL1 translocation (17–21). Ph-like ALL represents approximately 10% of all pediatric BCP-ALL cases and increases in incidence with age (17–21). Activating mutations in the pseudokinase or kinase domains of Janus Kinases (JAK) are observed in approximately 50% of Ph-like ALL cases, and are frequently accompanied by IKZF1 gene deletions. The presence of JAK mutations, IKZF1 deletions, and the Ph-like gene expression signature are associated with poor outcome (17, 20, 22, 23), highlighting the need for more targeted therapies.

Here we demonstrate that birinapant exerts potent antileukemic efficacy in vitro and in vivo against pediatric and adult BCP-ALL primary samples and patient-derived xenografts (PDX), in particular those derived from Ph-like ALL, via a TNFα-dependent process associated with high expression of TNFR1. We also identified a gene expression signature in BCP-ALL associated with birinapant response that was enriched in genes involved in inflammatory response, hematopoiesis, and cell death pathways. Birinapant also significantly enhanced the antileukemic activity of an induction-type regimen of vincristine, dexamethasone, and i-asparaginase (VXL) in vivo. These findings support the further clinical evaluation of birinapant in the treatment of BCP-ALL and other hematologic malignancies.

Materials and Methods

Primary ALL cells and PDXs

Human leukemia cells used in this study were from peripheral blood or bone marrow biopsy specimens of ALL patients. Methods to establish PDXs from pediatric BCP ALL biopsies in NOD/SCID (NOD.CB17-Prkdcscid/J) or NOD/SCID/IL-2 receptor gamma−/− (NOD.Cg-Prkdcscid/Il2rg−/−;Sz/Sz) NSG mice have been described previously (24–29). Patient demographics of PDXs and primary patient samples are described in Supplementary Table S1. ALLs were classified as Ph-like using gene expression criteria described previously (18–21, 30).

Apoptosis assays

PDX cells were resuspended in QBSF-60 medium (Quality Biological) with 20 ng/mL Flt-3 ligand, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine. Patient samples were cultured in StemSpan media (Stem Cell Technologies) with 20 ng/mL IL3, 20 ng/mL IL6, 100 ng/mL FLT3-L, and 100 ng/mL SCF. The NALM6 cell line (RIKEN BioResource Center) was used within 3 months of culture after validation by short tandem repeat analysis, and maintained in RPMI with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. For some experiments, cells were pretreated with 10 ng/mL TNFα (Lonza Australia), 10 μg/mL Enbrel (etanercept, soluble TNFR2 used to Fc; Clifford Hallam Healthcare), 10 μg/mL Q-VD-Oph (QVD; Sigma-Aldrich), or 10 μM/L Necrostatin-1 (Nec-1; Selleck Chemicals) for 2 hours before the addition of birinapant (TetraLogic Pharmaceuticals). Apoptosis was determined using Annexin V/7-AAD staining 24 hours later.

Protein expression analysis

PDX cells were treated with 1 μM/L birinapant for 0, 0.5, and 6 hours. Analysis of cellular proteins by immunoblotting was performed as described previously (31). Membranes were probed with specific antibodies for the following proteins: cIAP1 (7065S), cIAP2 (3130S), XIAP (2043S), RIP1 (4926S), caspase-8 (9746S), cleaved caspase-8 (8392S), caspase-3 (9662S), cleaved caspase-3 (9664S), TNFR1 (3736S; Cell Signaling Technology), and actin (A2066; Sigma-Aldrich). The secondary antibody used was horse-radish peroxidase (HRP) conjugates of anti-rabbit IgG (GE Healthcare). Signals were detected by Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and visualized using a VersaDoc 5000 Imaging System (Bio-Rad).

RNA extraction and RT-PCR

Total RNA was isolated from PDX cells using the RNeasy Mini Plus Kit (Qiagen). A high capacity cDNA reverse transcription kit (Applied Biosystems) was used to synthesize cDNA from 1 μg total RNA. TNFR1 mRNA expression was measured using TaqMan Gene Expression Assays (Hs01042313_m1; Applied Biosystems). EFla was used as an endogenous control and data are expressed as fold change relative to normal peripheral blood mononuclear cells (PBMC).

In vivo drug treatments

PDX cells were transplanted into 6- to 8-week-old female NOD/SCID or NSG mice of 20–25 g weight by intravenous injection. Leukemia engraftment was monitored by flow cytometric quantification of the proportion of human CD45-positive (huCD45+) cells versus total CD45+ leukocytes (human + murine) in the peripheral blood, bone marrow, or spleens as described previously (24, 26–29). When the median %huCD45+ cells in the peripheral blood was above 1% for the entire cohort, mice were randomized and allocated to treatment groups (7–9 mice per group). For single-agent efficacy studies, birinapant was administered via intraperitoneal (i.p.) injection at a dose of 30 mg/kg, every 3 days × 5. Where indicated, Enbrel was administered at 10 mg/kg i.p., 4 hours before birinapant treatment. For combination studies, birinapant was given twice a week for two weeks at 10 mg/kg. VXL (Prince of Wales Hospital Pharmacy, NSW, Australia) was administered Monday–Friday for two weeks via intraperitoneal injection, and consisted of vincristine (0.15 mg/kg) on Monday only followed by daily dexamethasone (5 mg/kg) and i-asparaginase (1,000 U/kg; ref. 32).

Determination of in vivo treatment response

Individual mouse event-free survival (EFS) was calculated as the time in days from treatment initiation until the %huCD45+ cells in the peripheral blood reached 25%, or until mice reached a humane endpoint with evidence of leukemia-related morbidity. EFS time was represented graphically by Kaplan–Meier analysis and survival curves were compared by log-rank test. The response to drug treatment was evaluated by two methods: (i) progression delay (T-C), calculated as the difference between the median EFS of the drug-treated cohort (T) and the median EFS of the vehicle-treated cohort (C); and (ii) using an objective response measure (ORM) modeled after stringent clinical criteria, which was assessed at day 42 after treatment initiation as described previously (33) and detailed in the Supplementary Methods.

In vivo pharmacodynamic analysis

Mice were inoculated with ALL PDX cells and monitored until peripheral blood engraftment reached >80% huCD45+, at which stage a single dose of birinapant (30 mg/kg) or vehicle was
administered. Groups of 3 mice were humanely killed and spleens harvested 6 hours after treatment. Mononuclear cells were isolated and samples confirmed to be >98% huCD45+ via flow cytometry. Protein expression was subsequently analyzed by immunoblotting.

Gene expression analysis

The raw expression data were preprocessed using Illumina BeadStudio (v2011.1) and Partek Genomics Suite (v6.6) including background subtractions, quantile normalization, and log2 transformation. The normalized expression levels were further processed using the ComBat (v.3) algorithm, implementing a parametric estimator with one covariate (e.g., the leukemia subtype) to remove potential batch effects. The batch-removed expression levels were analyzed using the ANOVA and 68 differentially expressed genes between responders and nonresponders were identified. The expression levels of the 68 genes within the birinapant-signature were scaled to a mean of zero and SD of one and hierarchical clustering average linkage method and the Euclidean distance measure was performed. Similarly, the 68 genes within the birinapant-signature were analyzed using the Ingenuity IPA Core Analysis algorithm (v.24390178) and canonical, cellular, developmental, and disease pathways, significantly associated with the molecular response to birinapant treatment, identified. Gene expression datasets can be accessed at www.ncbi.nlm.nih.gov/geo (accession no. GSE52991, GSE57795, GSE74460).

Statistical analysis

Quantitative data from in vitro assays and in vivo experiments were compared using the Mann–Whitney U test or Student's t test. Correlations were analyzed using Pearson correlation coefficient. P values <0.05 were considered significant. The exact log-rank test was used to compare EFS distributions between treatment and control groups (two-tailed), with P ≤ 0.05 considered significant. To evaluate interactions between drugs in vivo, therapeutic enhancement was considered if the EFS of mice treated with the combination treatment was significantly greater (P < 0.01) than those induced by both single arms of the study (34, 35).

Study approval

All studies had prior approval from the Human Research Ethics Committees of the University of New South Wales and the South Eastern Sydney Local Health District, and the Animal Care and Ethics Committee of the University of New South Wales. Written informed consent was received from participants prior to inclusion in the study.

Results

Birinapant exerts potent antileukemic efficacy against BCP-ALL PDXs and primary cells in vitro, and in particular Ph-like ALL

We have previously described the development and characterization of panels of PDXs representative of diverse pediatric ALL subtypes, including BCP-ALL, Ph-like ALL, infant MLL-ALL–rearranged ALL (MLL-ALL), T-ALL, and early T-cell precursor ALL (ETP-ALL; refs. 24–29), details of which can be found in Supplementary Table S1. As another small-molecule SMAC mimetic (BV6) had previously shown in vitro efficacy against pediatric ALL cell lines and primary samples (36) we wished to determine whether birinapant exhibited any subtype-specific efficacy. Therefore, we assessed the in vitro sensitivity of 41 PDXs after 24-hour exposure to 100 nmol/L birinapant (Fig. 1A). Using an arbitrary cutoff of 50% of control cell viability, 9 of 9 Ph-like ALL PDXs were sensitive to birinapant, compared with 7 of 13 BCP-ALL, 3 of 7 MLL-ALL, 1 of 9 T-ALL, and 1 of 3 ETP-ALL. When stratified according to B-lineage (BCP-ALL, Ph-like ALL, infant MLL-ALL) or T-lineage (T-ALL, ETP-ALL), the B subtype was significantly more sensitive than T-lineage (P < 0.001, Fig. 1B). Within the B-lineage ALL PDXs the Ph-like subtype was significantly more sensitive than other high-risk subtypes such as infant MLL-ALL (Fig. 1C). To verify that the sensitivity of Ph-like PDX cells was not an artifact of xenografting, we next tested the birinapant sensitivity of primary ALL cells derived from 6 pediatric and 3 adult patients with Ph-like ALL, alongside 3 BCP-ALL primary samples. Using the same cutoff as for the PDX cells, 9 of 9 primary Ph-like biopsies were sensitive to birinapant, and 2 of 3 non–Ph-like samples were resistant (Fig. 1D; Supplementary Table S1). The predisposition of Ph-like ALL PDXs and primary samples to birinapant sensitivity did not appear to be due to the presence of specific kinase lesions per se, as in the B-ALL cohort of PDXs and primary biopsies kinase lesions were detected in 4 of 8 sensitive and 3 of 8 resistant samples (Fig. 1A and D; Supplementary Table S1). Moreover of the 4 BCR-ABL1–positive samples, 2 were sensitive (ALL-56 and #110) while 2 were resistant (ALL-4 and ALL-55).

To further investigate the mechanism by which birinapant kills Ph-like ALL cells, PDXs were preincubated with the pan-caspase inhibitor QVD or the RIPK1 inhibitor Nec-1 prior to birinapant treatment. QVD significantly attenuated birinapant-induced apoptosis in 3 of 4 sensitive PDXs, while Nec-1 caused significant inhibition in 2 of 4 PDXs (Fig. 1E). These results indicate that birinapant kills Ph-like ALL cells via caspase- and RIPK-mediated pathways.

Both cIAP1 and cIAP2 were rapidly degraded in sensitive (n = 3) and resistant (n = 2) PDXs within 30 minutes of birinapant treatment (Fig. 1F). The cleaved (activated) forms of both caspase-3 and caspase-8 were markedly upregulated in sensitive PDXs within 6 hours of birinapant treatment in contrast to the resistant PDXs, indicating that resistance occurred downstream of cIAP1/2 degradation. Consistent with the decreased ability of birinapant to inhibit XIAP-dependent signaling pathways compared with cIAP1/2 (8), XIAP protein levels only markedly decreased in the 3 sensitive PDXs after 6 hours of birinapant treatment concomitant with caspase activation, suggesting that XIAP degradation is a consequence, rather than a cause, of cell death.

The sensitivity of BCP-ALL to birinapant is replicated in vivo

When administered at a dose of 30 mg/kg, birinapant induced significant progression delays in 17 of 19 PDXs relative to control vehicle–treated mice, with progression delays (T-C) ranging from 2 to 76.9 days (Supplementary Table S2). Representative plots of leukemia progression over time and corresponding Kaplan–Meier survival plots illustrating Ph-like PDX responses to birinapant are shown in Fig. 2A, which indicate that prolonged extension of mouse EFS could be induced despite a brief treatment window (12 days). Using stringent objective response criteria modeled after the clinical setting (33), 11 of 19 PDXs achieved objective responses (1 partial response, PR; 7 complete responses, CRs; 3 maintained CRs, MCRs, Supplementary Table S2; Fig. 2B and C and Supplementary Figs. S1 and S2), including 6 of 6 Ph-like ALL.
PDXs. The birinapant treatments were well tolerated and caused no significant decrease in body weight or perturbations in hemato logic parameters (Supplementary Fig. S3). A complete summary of results is provided in Supplementary Table S3. Of note, only 1 of 165 (<1%) of birinapant-treated mice experienced toxicity-related events.

Similar to the in vitro data described above, the Ph-like PDXs were significantly more sensitive to birinapant in vivo compared with the BCP-ALL and MLL-ALL PDX panels, when assessed by EFS T-C (Fig. 2D) or T/C values (Fig. 2E). Moreover, the in vitro birinapant sensitivity (% of control after treatment with 100 nmol/L) of the 19 PDXs tested significantly correlated with their in vivo responses (T-C; R² = 0.41, P < 0.005; Fig. 2F). Consistent with the in vitro results, birinapant also caused rapid (within 6 hours) degradation of cIAP1 regardless of the PDX response (Fig. 2G), despite clear differences in caspase-3, -7, -8, and -9 activation between a sensitive (ALL-2) and resistant (ALL-7) PDX.

Birinapant induces significant regressions in Ph-like ALL PDXs over a broad dose range and demonstrates synergy with established drugs

A desirable characteristic for an anticancer drug is that it is effective over a broad dose range and not only at its MTD. To test this quality for birinapant, we treated mice engrafted with 2 Ph-like PDXs with doses as low as 1/8th of its MTD (30 mg/kg). Birinapant significantly delayed the progression of both PAMDRM and PALLSD at all doses tested, resulting in significantly increased EFS of mice across all treatment groups (Fig. 3A; Supplementary Table S2). The efficacy of birinapant was dose-dependent, with T-C values of 24.6–64.7 days for PAMDRM and 5.5–24.9 days for PALLSD. Objective responses were achieved at all 4 dose levels for PAMDRM and at the 2 highest doses for PALLSD (Supplementary Table S2).

Assessment of PAMDRM at days 0 and 14 revealed a profound and significant reduction in leukemia infiltration in all organs at all 4 dose levels tested (Fig. 3B), with 97% clearance of human
leukemia cells. These results demonstrate consistency in measuring antileukemic drug responses by assessing the peripheral blood, bone marrow, or spleen, and that birinapant can induce significant leukemia regressions over a broad dose range.

An additional desirable quality for an anticancer drug is that it enhances the efficacy of established therapy, or in the least is not antagonistic. This is particularly relevant in pediatric ALL, in which effective combination chemotherapy treatments are available for the vast majority of patients. Mice engrafted with PAKHZT and PALLSD were subjected to a two-week treatment schedule combining birinapant with an induction-type regimen of VXL (32). Both VXL and birinapant (10 mg/kg, 1/3rd its MTD) significantly delayed the progression of PAKHZT and PALLSD compared with vehicle controls (Fig. 4A; Supplementary Table S4). Birinapant achieved progressive disease 2 (PD2) in PALLSD (P < 0.0001) and a CR in PAKHZT (P = 0.001), while VXL achieved CRs in both PDXs. The combination of VXL plus birinapant significantly extended disease remission to 35.3 and 40.9 days for PALLSD and PAKHZT, respectively, elicited CRs and resulted in therapeutic enhancement for both PDXs.

Because of the efficacy of conventional treatment regimens in pediatric ALL, any phase I/II clinical trial of birinapant is likely to focus on relapsed/refractory patients. Therefore, we have emphasized the preclinical testing of birinapant against PDXs derived from high-risk pediatric ALL subgroups, or from patients who have relapsed (Supplementary Table S1). To directly test the in vivo efficacy of birinapant on previously treated ALL PDXs, mice engrafted with PAKHZT were treated with birinapant after they had relapsed with >25%CD45+ cells in the peripheral blood after prior VXL or birinapant treatment (Fig. 4B–F). Birinapant (10 mg/kg) was highly effective in reducing high leukemic burden in the peripheral blood, bone marrow and spleens of mice that had been pretreated with either regimen, indicating that birinapant is likely to be effective against relapsed disease.

A unique gene expression signature distinguishes birinapant sensitive and resistant PDXs

To evaluate a potential molecular predisposition for birinapant response in BCP-ALL, we interrogated gene expression profiles of 12 BCP-ALL PDXs. ANOVA identified that a signature of 68 genes was differentially expressed between responders and nonresponders in BCP-ALL (birinapant-signature, Fig. 5A; Supplementary Table S5). Hierarchical clustering of the birinapant-signature across the 12 BCP-ALL PDXs confirmed that these samples were optimally separated into two major classes corresponding with response to birinapant treatment (Fig. 5A). Importantly, hierarchical clustering of the same BCP-ALL PDXs but with an additional 6 Ph-like PDXs (PAKHZT, PAKSWW, PAKSRL, ALL-10, PAMDRM, and PALLSD) confirmed that the Ph-like PDXs, consistent with their functional response, all grouped with other birinapant responders (Fig. 5B and C). Ingenuity Pathway Analysis (IPA) further revealed that the birinapant-signature had the highest enrichment for genes associated with inflammatory response (P = 3.48E-06-6; disease pathways), Hematologic system development and function (P = 6.03E-07; developmental pathways) and cell death and survival (P = 3.22E-06, cellular pathways; Fig. 5D). When visualized using the ‘organic’ layout algorithm, the birinapant-signature was enriched for components of the TNF receptor signaling pathway including up-regulation of TNFR1 and its direct targets NOS2 and IL12A in responders (Fig. 5E; Supplementary Table S5). Strikingly, when visualized using the ‘hierarchical’ layout algorithm TNFR1 was identified as the founder node and major regulator of the underlying network (Supplementary Fig. S4).

Validation of an essential role for the TNF receptor pathway in birinapant-induced death of ALL PDXs and primary cells

We next sought to validate the role of the TNF receptor pathway in the mode of action of birinapant in ALL PDXs and primary samples. Both TNFR and TNFR1 gene expression significantly correlated with in vivo sensitivity to birinapant (Fig. 6A and B). Moreover, TNFR1 mRNA and protein were expressed at moderate to high levels in 5 of 5 birinapant-sensitive PDXs (Fig. 6C and D), while 2 of 3 resistant PDXs expressed low to undetectable levels. Consistent with the differential expression of TNFR1 between the 3 birinapant-resistant PDXs (ALL-55, ALL-4, and -7) exogenous TNFα was unable to sensitize resistant xenografts to birinapant, but was cytotoxic by itself to ALL-55 (Fig. 6F).

Plasma from mice engrafted with ALL was next evaluated for TNF levels. Birinapant (10 mg/kg for 6 hours) significantly increased TNFα production in 4 of 4 PDX models, including 2 resistant xenografts, with no detectable levels present in control bearing animals (Fig. 6F). Like many cell lines, NALM6 cells only responded to birinapant in the presence of exogenous TNFα (Fig. 6G), which is clearly not the case for primary ALL cells and PDXs (Fig. 1A and D). To ensure that the culture media was not contributing to birinapant cytotoxicity, both NALM6 and a Ph-like PDX PAMDRM (Fig. 6G) were cultured in RPMI and QBSF media prior to treatment. No significant differences in response were observed for either cell type regardless of the media used. These data suggest that any potential trace amounts of TNFα present in the media are insufficient to induce birinapant-mediated cell death in ALL PDXs.

We next showed that Enbrel, a clinical-grade soluble TNF receptor fused to Fc, offered almost complete protection against birinapant-induced cell death of ALL PDXs and primary cells in vitro (Fig. 7A and B). More importantly, Enbrel inhibited the antileukemic effects of birinapant against the Ph-like PAKHZT PDX in vivo. Birinapant alone profoundly reduced leukemic infiltration in the murine peripheral blood (<0.2%), bone marrow (<5%), and spleens (<1%) at day 14 after treatment initiation (Fig. 7C), and substantially delayed leukemia progression (Fig. 7D and E) and induced a CR (Supplementary Table S4). In contrast, Enbrel cotreatment resulted in leukemia persistence in the bone marrow, spleens, and peripheral blood (Fig. 7C), and significantly accelerated disease relapse (Fig. 7D and E; Supplementary Table S4).

**Discussion**

The IAP family of proteins represents a promising therapeutic target in pediatric ALL due to the fact that overexpression can contribute to evasion of apoptosis. In 2011, The Pediatric Preclinical Testing Program (PPTP) reported that LCL-161, a monovalent IAP inhibitor, was ineffective against a panel of pediatric ALL PDXs (37). Birinapant, however, is reported to be superior to other IAP inhibitors, primarily due to its bivalent structure and ability to target multiple IAP proteins simultaneously (8). Here we report that the majority of a large panel of BCP-ALL PDXs is sensitive to birinapant in vitro at submicromolar concentrations, including 9 of 9 Ph-like BCP-ALL PDXs. Furthermore, 100% of primary Ph-like ALL samples were also sensitive to birinapant.
confirming that the sensitivity of Ph-like subtype was not an artifact of xenografting.

Overall, these findings are of particular interest given that many previous studies have reported that SMAC mimetics are ineffective against primary tumor cells when administered as a single agent, with the majority of cells being resistant or only being effected at high micromolar concentrations (38-41). In addition, we have demonstrated significant therapeutic efficacy of birinapant against models of high-risk, aggressive or chemoresistant disease in vivo, a property that lends itself to the use of birinapant in the relapsed/refractory disease setting.

Figure 2.
Birinapant exhibits significant single-agent efficacy against Ph-like BCP-ALL PDXs in vivo. A, individual %huCD45+ cells in the peripheral blood (PB) of mice and Kaplan–Meier curves for EFS (bottom) for 19 PDX lines treated with birinapant. B, distributions of objective response measures of individual mice from 19 PDX lines treated with birinapant. Black boxes on the x-axis indicate treatment days. B, distributions of objective response measures of individual mice from 19 PDX lines treated with birinapant. Black boxes on the x-axis indicate treatment days. B, distributions of objective response measures of individual mice from 19 PDX lines treated with birinapant. Black boxes on the x-axis indicate treatment days. B, distributions of objective response measures of individual mice from 19 PDX lines treated with birinapant. Black boxes on the x-axis indicate treatment days. B, distributions of objective response measures of individual mice from 19 PDX lines treated with birinapant. Black boxes on the x-axis indicate treatment days.

Figure 3.
Birinapant exhibits in vivo efficacy over a broad dose range. A, individual and median (left and middle panels) %huCD45+ cells in the peripheral blood (PB) of mice and Kaplan–Meier curves for EFS (right) for 2 Ph-like BCP-ALL PDXs, PALLSD, and PAMDRM. Black boxes on the x-axis indicate treatment days. B, assessment of disease dissemination in the peripheral blood, bone marrow, and spleen of the Ph-like xenograft PAMDRM, 14 days after treatment initiation. Data represent the mean ± SEM, n ≥ 3 mice/group. *P < 0.05, **P < 0.001. Student t test.

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The clinical development of any potential new agent may be impaired if the drug is only effective at its MTD. Here we have shown that birinapant was still able to induce significant leukemia regressions in vivo when administered at 1/8th of its MTD, not only clearing blasts from the peripheral blood but also from the major hematopoietic organs including the spleen and bone marrow. Moreover, the significant antileukemic efficacy observed in this study was achieved using doses of birinapant that are not only well tolerated in mice but are also reflective of drug levels attainable in humans (42).

Despite the high overall survival rate for pediatric ALL, treatment outcomes remain poor for the approximately 20% of patients who experience relapse. Attempts to improve outcomes of primary high-risk disease by intensification of existing treatment, while largely successful, carry the burden of high toxicity (43, 44), thus highlighting the need for targeted approaches.
Figure 5.
Generation of a birinapant sensitivity gene expression signature. A, hierarchical clustering of 12 BCP-ALL PDX samples (columns), based on the pretreatment expression levels of the birinapant-signature genes (rows), grouped as responders (green) or nonresponders (red). B, hierarchical clustering of the same 12 PDXs plus an additional 6 Ph-like PDXs showing that the birinapant-signature was maintained in Ph-like ALL responders. C, principal component analysis of the same samples identified that the first principal component was sufficient to separate responders (blue) from nonresponders (red). D, IPA of the birinapant-signature identified the most enriched pathways associated with developmental, diseases, and cellular expressed as $-\log(P\text{ value})$. E, network analysis (IPA) of the birinapant-signature using the organic layout algorithm identified a network centered on TNFR1.
therapies that could be incorporated to strengthen current combination chemotherapy regimens. Similar to a recent study using BV6 (45), combining birinapant with an induction-type regimen (VXL) significantly enhanced objective responses and prolonged leukemia regressions in two Ph-like PDXs in vivo. Importantly, no additional side effects were observed when birinapant was combined with VXL combination therapy in vivo. Furthermore, we have clearly demonstrated that birinapant remains effective against leukemia cells previously exposed to either standard chemotherapy (VXL) or birinapant in vivo, highlighting a potential use for birinapant in the relapsed/refractory ALL clinical setting.

We next carried out an unbiased analysis of global gene expression profiles and identified a set of 68 genes associated with birinapant sensitivity in BCP-ALL. This gene set was distinct from that identified by others to classify Ph-like ALL (18, 20, 21, 30), and was enriched in genes associated with the TNFR1 pathway. Further evaluation of gene expression data revealed a significant correlation between TNFα expression and in vivo response to birinapant. There appears to be an absolute requirement for TNFα (or other inflammatory cytokines, i.e., TRAIL) for birinapant to exert its effect in both oncology and infectious disease settings (46, 47). Birinapant efficacy against PDXs and primary ALL patient samples was TNFα dependent, as demonstrated by reversing drug-induced cell death using Enbrel. Nevertheless, the source of TNFα in ALL PDXs and primary cells remains unclear and may be due to autocrine production; perhaps stimulated by birinapant treatment itself (40, 41, 48). Not surprisingly, expression of TNFR1 appeared necessary, but not sufficient, for birinapant sensitivity in pediatric ALL cells, with high level expression detected across the Ph-like panel.

Similar to bortezomib and topotecan (49, 50), birinapant showed significant single-agent efficacy against the PPTP ALL
PDX models. As bortezomib and topotecan also both showed significant clinical efficacy against pediatric ALL when combined with an induction-type regimen (51, 52), it can be predicted that birinapant would be highly efficacious in the management of relapsed/refractory ALL patients in combination with established drugs.

In summary, we have demonstrated that birinapant at clinically relevant doses displays profound antileukemic activity as a single agent and in combination with chemotherapy in PDX models of high-risk pediatric ALL, including Ph-like ALL. Birinapant exhibited superior efficacy against BCP-ALL compared with T-ALL in a large panel of PDXs, with Ph-like ALL the most sensitive BCP-ALL subtype. The identification of a gene expression signature associated with birinapant sensitivity may lead to biomarker-driven clinical trials to identify ALL patients who might benefit from birinapant treatment. Overall, birinapant appears to be a promising new drug for the treatment of high-risk pediatric BCP-ALL, and further clinical investigations into its potential applications appear warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Authors' Contributions
Conception and design: J. Richmond, H. Carol, P.J. Houghton, M.A. Smith, R.B. Lock
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References
Birinapant Sensitivity of B-cell Precursor ALL

Acute Sensitivity of Ph-like Acute Lymphoblastic Leukemia to the SMAC-Mimetic Birinapant

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