Survival of infants with KMT2A-rearranged acute lymphoblastic leukemia (ALL) remains dismal despite intensive chemotherapy. We observed constitutive phosphorylation of spleen tyrosine kinase (SYK) and associated signaling proteins in infant ALL patient-derived xenograft (PDX) model specimens and hypothesized that the SYK inhibitor entospletinib would inhibit signaling and cell growth in vitro and leukemia proliferation in vivo. We further predicted that combined entospletinib and chemotherapy could augment anti-leukemia effects. Basal kinase signaling activation and HOXA9/MEIS1 expression differed among KMT2A-rearranged (KMT2A-AFF1 [n=4], KMT2A-MLLT3 [n=1], KMT2A-MLLT1 [n=4]) and non-KMT2A-rearranged [n=3] ALL specimens and stratified by genetic subgroup. Incubation of KMT2A-rearranged ALL cells in vitro with entospletinib inhibited methylcellulose colony formation and SYK pathway signaling in a dose-dependent manner. In vivo inhibition of leukemia proliferation with entospletinib monotherapy was observed in RAS-wild-type KMT2A-AFF1, KMT2A-MLLT3, and KMT2A-MLLT1 ALL PDX models with enhanced activity in combination with vincristine chemotherapy in several models. Surprisingly, entospletinib did not decrease leukemia burden in two KMT2A-AFF1 PDX models with NRAS or KRAS mutations, suggesting potential RAS-mediated resistance to SYK inhibition. As hypothesized, superior inhibition of ALL proliferation was observed in KMT2A-AFF1 PDX models treated with entospletinib and the MEK inhibitor selumetinib versus vehicle or inhibitor monotherapies (P<0.05). In summary, constitutive activation of SYK and associated signaling occurs in KMT2A-rearranged ALL with in vitro and in vivo sensitivity to entospletinib. Combination therapy with vincristine or selumetinib further enhanced treatment effects of SYK inhibition. Clinical study of entospletinib and chemotherapy or other kinase inhibitors in patients with KMT2A-rearranged leukemias may be warranted.

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ferase 2A (KMT2A, formerly mixed lineage leukemia [MLL]; located at chromosome 11q23) are at higher risk of relapse and have inferior overall survival. Wild-type KMT2A rearrangements occur in approximately 10% of childhood and adult B-ALL cases with highest frequency (75%) in infants diagnosed with leukemia at <365 days old. Children with KMT2A-rearranged (KMT2A-R) ALL have a poor prognosis with 5-year event-free survival (EFS) of 20-50% in infants and approximately 58% in older children. Age <6 months at diagnosis, hyperleukocytosis with white blood cell count >300x10^9/L, and poor response to prednisone prophase chemotherapy have been associated with worst clinical outcomes and dismal long-term survival amongst infants with KMT2A-R ALL. Adults with KMT2A-R ALL have similarly poor outcomes with <50% 5-year EFS.

Wild-type KMT2A is required for normal hematopoiesis and post-natal hematopoietic cell maintenance. Disruption of KMT2A via chromosomal translocation in acute lymphoid and myeloid leukemias was first described nearly three decades ago. In ALL, these translocations result in fusion of KMT2A to one of >100 currently known translocation partner genes, leading to production of fusion proteins which disrupt normal regulation of gene expression by wild-type KMT2A. Recruitment of the super elongation complex (SEC) and the H3K79 histone methyltransferase DOT1L by the fusion proteins consequently leads to new fusion-dependent functions of KMT2A. While numerous partner genes have been reported, five translocations account for the majority of KMT2A rearrangements in ALL across the age spectrum. These include t(4;11)(q21;q23) with KMT2A-ALLF1 fusion (60%), t(11;19)(q23;p13.3) with KMT2A-MLLT1 fusion (18%), t(9;11)(p21;q23) with KMT2A-MLLT3 fusion (12%), t(10;11)(p12;q23) with KMT2A-MLLT10 fusion (3%), and t(6;11)(q27;q25) with KMT2A-MLLT4 fusion (1%).

Preclinical studies of murine models and primary patient specimens demonstrate that KMT2A-R ALL cells harbor gene expression signatures with distinct arrest in B-cell development at the pro-B and pre-B cell stages. Recent publications have reported a strong link between increased expression of the HOX cluster of transcription factor genes (particularly HOXA9) and its co-factor MEIS1 in accelerating KMT2A-R leukemia development via upregulation of spleen tyrosine kinase (SYK), as well as constitutive activation of SYK signaling in several B-ALL subtypes. There specific mechanisms by which KMT2A translocations contribute to SYK signaling in B-ALL and their role in leukemogenesis and maintenance have not been completely characterized.

SYK is expressed in hematopoietic cells and involved in multiple signal transduction pathways downstream of the B-cell receptor (BCR). SYK is autophosphorylated and activated when its two tandem Src homology 2 (SH2) domains bind to immunoreceptor tyrosine based activation motifs (ITAM). This binding then initiates downstream signal transduction via activation of effector molecules, including phospholipase C gamma (PLCγ), B-cell linker protein (BLNK), phosphatidylinositol 3 kinase (PI3K), and mitogen activated protein kinase (MAPK) that converge to activate multiple downstream signaling pathways involved in B-cell malignancies. This makes SYK an attractive potential therapeutic target. In vitro and in vivo activity of SYK inhibition in preclinical B-ALL models has been previously established and several SYK inhibitors (e.g., entospletinib, fostamatinib) are under evaluation in patients with relapsed/refractory solid tumors, hematologic malignancies, or autoimmune diseases.

Entospletinib (ENTO, formerly GS-9973) is a potent and highly selective SYK inhibitor under current clinical investigation in adults with relapsed acute leukemias (clinicaltrials.gov identifiers: NCT02343939 and NCT02404220). Interim analysis of a phase Ib/II study of ENTO and chemotherapy showed complete responses in two patients with relapsed KMT2A-R acute myeloid leukemia (AML) treated with ENTO monotherapy for 14 days, suggesting potential for particular clinical activity in KMT2A-rearranged leukemias. Translating the efficacy of SYK inhibition with ENTO and depth of response in combination with standard-of-care chemotherapy agents warrants further investigation at a molecular level. In the current study, we assessed the therapeutic potential of ENTO monotherapy and in combination with chemotherapy or other kinase inhibitors in preclinical infant KMT2A-R and non-KMT2A-R ALL patient-derived xenograft (PDX) models to delineate the potential anti-leukemic utility of SYK inhibition in this high-risk childhood leukemia subtype.

Methods

KMT2A-rearranged acute lymphoblastic leukemia patient specimens and xenotransplantation models
Viably cryopreserved leukemia cells from infants with de novo KMT2A-R (n=4; corresponding relapse, n=3) and non-KMT2A-R ALL (n=5) enrolled on the Children’s Oncology Group (COG) trial AALL0681 were obtained via informed consent as previously described. Additional specimens from an infant with relapsed KMT2A-R (n=1; ALL3108) and an adult with de novo KMT2A-R ALL (n=1; ALL3113) were obtained from the University of California, San Francisco and University of Pennsylvania leukemia biorepositories under approved institutional research protocols after informed consent in accordance with the Declaration of Helsinki (Table 1). PDX models were established in NOD.Cg-Prkdcscid IScg1Ifnγ−/− /Slc/HrNSG (NSG) mice via an Institutional Animal Use and Care Committee-approved protocol at the Children’s Hospital of Philadelphia as described with serial transplantation of human ALL cells into secondary or tertiary recipients for experimental studies. Additional established non-KMT2A-R ALL PDX models (primarily of the Philadelphia chromosome-like [Ph-like] subtype) were used as negative controls.

Kinase inhibitors and chemotherapy
The selective SYK inhibitor entospletinib (ENTO) was provided as a dispersible powder for in vitro studies and in rodent chow formulation in 0.03%, 0.05%, and 0.07% concentrations for in vivo animal studies by Gilead Sciences Inc. (Foster City, CA, USA). Rodent chow concentrations were selected and optimized based upon PK levels achieved in ENTO-treated adult patients with acute leukemia (clinicaltrials.gov identifiers: NCT02404220 and NCT02343939). Vincristine and dexamethasone were purchased from the Children’s Hospital of Philadelphia investigational pharmacy (Philadelphia, PA, USA). The MEK inhibitor selumetinib, SYK inhibitor fostamatinib, and multi-kinase inhibitor dasatinib were purchased from Selleckchem (Houston, TX, USA) or LC Labs (Woburn, MA, USA). Cell viability and phosphoflow cytometry...
Further details about described. Vincristine dosing was previously optimized in ALL studies) or up to 28 days (treatment efficacy studies) as vincristine for 72 hours (pharmacokinetic [PK] and pharmacodynamic

- Peripheral blood, engrafted ALL PDX models were randomized to chromosome-conjugated antibodies from EBioscience) in murine
- Results

### Table 1. Molecular and cytogenetic characteristics of acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) models.

<table>
<thead>
<tr>
<th>ALL PDX model</th>
<th>COG USI</th>
<th>KMT2A status</th>
<th>Translocation</th>
<th>Disease status</th>
<th>Other genetic alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL185GD</td>
<td>PAWRRD</td>
<td>wild-type</td>
<td>P2RYR-CRLF2, PA5-AKT2</td>
<td>De novo</td>
<td>JAK2 mut, CDKN2A/B del</td>
</tr>
<tr>
<td>ALL31GD</td>
<td>PAUFHC</td>
<td>wild-type</td>
<td>P2RYR-CRLF2, PA5-C20orf112</td>
<td>De novo</td>
<td>JAK2 del, CDKN2A/B del, RTEL del</td>
</tr>
<tr>
<td>ALL32GD</td>
<td>PAUXSA</td>
<td>wild-type</td>
<td>t(1;19) (q23p13) with TCF3-PBXI</td>
<td>De novo</td>
<td>KRAS mut, WHSC1 mut, gain CCND3, MYB, ESR1</td>
</tr>
<tr>
<td>ALL50MD</td>
<td>PAWEDG</td>
<td>KMT2A-AFF1</td>
<td>t(4;11) (q21q23)</td>
<td>De novo</td>
<td>KRAS mut</td>
</tr>
<tr>
<td>ALL42MD</td>
<td>PAVRBV</td>
<td>KMT2A-AFF1</td>
<td>t(4;11) (q21q23)</td>
<td>De novo</td>
<td>NRG1 mut</td>
</tr>
<tr>
<td>ALL42MR</td>
<td>PAVRBV</td>
<td>KMT2A-AFF1</td>
<td>t(4;11) (q21q23)</td>
<td>Relapse</td>
<td>NRG1 mut, IKZF1 del, cnLOH of chr22</td>
</tr>
<tr>
<td>ALL133MR</td>
<td>n/a</td>
<td>KMT2A-AFF1</td>
<td>t(4;11) (q21q23)</td>
<td>De novo</td>
<td>JAK2 mut, TP53 1p del, IKZF1 1p del</td>
</tr>
<tr>
<td>ALL153MR</td>
<td>PAUXIT</td>
<td>KMT2A-MLLT3</td>
<td>t(9;11) (q21q23)</td>
<td>Relapse</td>
<td>None identified</td>
</tr>
<tr>
<td>ALL155MR</td>
<td>PAUXIT</td>
<td>KMT2A-MLLT1</td>
<td>t(11;19) (q23p13)</td>
<td>De novo</td>
<td>None identified</td>
</tr>
<tr>
<td>ALL18MD</td>
<td>PAHSVFM</td>
<td>KMT2A-MLLT1</td>
<td>t(11;19) (q23p13)</td>
<td>Relapse</td>
<td>None identified</td>
</tr>
<tr>
<td>ALL26MR</td>
<td>PAHSVFM</td>
<td>KMT2A-MLLT1</td>
<td>t(11;19) (q23p13)</td>
<td>Relapse</td>
<td>Partial 1q del, including PTEN</td>
</tr>
</tbody>
</table>

**COG USI:** Children’s Oncology Group unique specific identifier; cnLOH: copy-neutral loss of heterozygosity; del: deletion; mut: mutation; n/a: not available.

- In vivo drug testing in patient-derived xenograft models

Animal studies were conducted under a CHOP Institutional Animal Use and Care Committee (IACUC)-approved protocol in accordance with the Panel on Euthanasia of the American Veterinary Medical Association’s guidelines. After flow cytometric (FC) confirmation of ≥1% CD45+ CD19+ human ALL (fluorochrome-conjugated antibodies from EBioscience) in murine peripheral blood, engrafted ALL PDX models were randomized to treatment with vehicle, ENTO chow orally ad libitum, vincristine 0.1 mg/kg intraperitoneally (IP) weekly, or both ENTO and vincristine for 72 hours (pharmacokinetic [PK] and pharmacodynamic [PD] studies) or up to 28 days (treatment efficacy studies) as described.
- Vincristine dosing was previously optimized in ALL cell line and PDX models (not shown). Additional studies in some ALL PDX models assessed selumetinib 100 mg/kg administered orally twice daily 5 days/week as (ALL155MR and ALL3113) or dexamethasone 1 mg/kg PO once daily 5 days/week (ALL3113, ALL38GD) as monotherapy or in combination with ENTO. Further details about in vivo drug testing in ALL PDX models and conduct of all other experimental studies are included in the Online Supplementary Methods.

**Results**

Characterization of constitutive SYK pathway activation in infant KMT2A-R acute lymphoblastic leukemia patient-derived xenograft models

Constitutive SYK pathway activation was detected across a genetic spectrum of infant ALL and some non-infant Philadelphia chromosome-like (Ph-like) ALL control specimens using harvested murine spleens from well-engrafted PDX models (Table 1). Assessment of phosphorylated and total SYK levels revealed that expression of high basal phosphorylated SYK (pSYK) was seen in the majority of infant non-KMT2A-R and KMT2A-R ALL specimens (Figure 1, left). pSYK levels were also elevated in some Ph-like ALL specimens and absent in splenic tissue from non-leukemia-injected NSG mice (Figure 1, right). Total SYK expression was relatively consistent across all models. The observed constitutive basal pSYK levels, coupled with a previously suggested role of upregulated SYK as a driver in AML models with high levels, coupled with a previously suggested role of upregulated SYK as a driver in AML models with high HOXA9 and MEIS1 expression, and early reports of clinical responses in adults with relapsed KMT2A-R leukemias treated with entospletinib led us to investigate the role of SYK signaling and therapeutic potential of ENTO specifically in infant KMT2A-R ALL PDX models.

Entospletinib decreases leukemic burden and inhibits kinase signaling in KMT2A-R acute lymphoblastic leukemia

SYK plays a pivotal role upstream of several key leukemia-associated signaling pathways, including RAS/RAF/MAPK, PI3K/AKT/mTOR, and JAK/STAT. SYK inhibition by ENTO has the potential to impact multiple signal transduction pathways in ALL (Visual Abstract), leading to potential anti-leukemic efficacy. Given our initial demonstration of constitutive SYK and other signaling pathway activation in infant ALL specimens by Simple Western, we first assessed leukemia cell growth inhibitory effects of ENTO in vitro using methylcellulose colony assays. Viably cryopreserved harvested KMT2A-R PDX ALL cells (model ALL3103 with KMT2A-MLLT3 fusion) were grown under anchorage-independent (non-adherent) conditions in serum-free methylcellulose and treated with a clinically-relevant dose range of ENTO for 14 days (Figure 2A). ENTO maximally inhibited colony formation (89% inhibition; P<0.0001 by t-test), suggesting that SYK plays a central role upstream of signaling pathways essential to proliferation and survival.

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We then assessed the ability of ENTO to inhibit leukemia proliferation in vivo in ALL3108 and NH011 (Ph-like ALL with NUP214-ABL1 fusion) PDX mice. ENTO 0.03% and 0.07% chow concentrations administered for 28 days both potently decreased human CD45⁺ CD19⁺ ALL cell counts in peripheral blood measured weekly by quantitative flow cytometry and in end-study spleens (Figure 2B and C and Online Supplementary Figure S7). Terminal PK evaluation of ENTO in the periphery confirmed that high levels of ENTO could be achieved by continuous chow administration (Figure 2D) without statistical difference between the 0.03% and 0.07% treatment groups. Simple Western analysis of highly leukemia-engrafted splenic lysates from individual ENTO-treated mice demonstrated marked inhibition of pSYK Y323, cMYC and pERK T202/Y204 as compared to control chow-treated animals after 4 weeks of treatment (Figure 2E) and high correlation between ENTO levels and pSYK and pERK inhibition in well-engrafted ALL3108 PDX mice treated in pharmacodynamic studies for 72 hours with entospletinib (Online Supplementary Figure S8). These results confirmed the on-target inhibition of pSYK and key downstream signaling phosphoproteins by ENTO, suggesting that an achieved dose level of 3330-7900 nM in vivo was sufficient to inhibit constitutive pSYK signaling and decrease in vivo leukemia proliferation in an aggressive relapsed infant KMT2A-R ALL PDX model.

**In vitro pharmacodynamic inhibition of signaling proteins in infant KMT2A-R models**

To extend our observation of ALL cell SYK dependency for proliferation and survival in other KMT2A-R fusion types, we evaluated ENTO in another aggressive multiply-relapsed infant ALL PDX model with KMT2A-MLLT1 fusion (ALL135MR) in short-term in vitro cultures and observed dose-dependent inhibition of pERK1/2, pAKT³⁴⁺, pSTAT5, and cMYC (Figure 3A). Interestingly, similar in vitro incubation of leukemia cells from an infant ALL PDX model with KMT2A-AFF1 fusion and concomitant NRASG12D mutation (ALL142MR) with ENTO showed little to no inhibition of the same key pathways (Figure 3B). These data suggest differential signaling effects potentially related to specific KMT2A fusion partner and/or RAS-mutant status.

**Evaluation of expression signatures in KMT2A-R acute lymphoblastic leukemia subtypes**

KMT2A-R ALL has been shown to have distinct gene expression signatures that define B-cell developmental arrest at either the pro-B- and pre-B-cell stages.22 Understanding the signaling pathway dependencies of different KMT2A-R fusion proteins in infant ALL cells may lead to more effective therapeutic targeting strategies for this high-risk patient population. To assess potential differential gene expression signatures, we evaluated the transcription factors HOXA9 and MEIS1, which are known downstream targets of KMT2A. As hypothesized, HOXA9 and MEIS1 expression levels correlated with both KMT2A-R fusion status and specific gene partner (Figure 4A). Infant ALL specimens with KMT2A-MLLT3 and KMT2A-MLLT1 fusions expressed both high HOXA9 and MEIS1, while KMT2A-AFF1 models had high MEIS1 and normal HOXA9 expression. Conversely, infant non-KMT2A-R samples had normal expression levels of HOXA9 and MEIS1. These distinct expression signatures exhibited amongst KMT2A-R samples with different fusion partners are concordant with reports of differential chromatin binding of KMT2A-R fusion proteins leading to distinct gene expression profiles and potentially differential clinical outcomes.23,24

Given the observed stratification of HOXA9 and MEIS1 expression signatures among the KMT2A subgroups, we next assessed protein expression signatures in these samples to evaluate potential correlation. Simple Western analysis of splenic lysates from KMT2A-R and non-KMT2A-R ALL PDX models (Figure 4B) demonstrated that leukemias with different KMT2A fusion partners induced different patterns of signaling activation. High levels of cMYC were detected only in KMT2A-AFF1 models, while KMT2A-MLLT1 models had high SRC, absent PTEN, and high pAKT levels. Regulation of both SRC and PI3K pathways are known to be potentially SYK-dependent, concordant with data from our in vitro studies in ENTO-treated ALL135MR cells (Figure 3A). Overall, differential gene expression signatures between KMT2A-R and non-KMT2A-R ALL subtypes (Online Supplementary Figure S9A) and differences between gene and protein expression signatures among the KMT2A fusion subtypes (Figure 4B and Online Supplementary Figure S9B) showed unique signaling...
dependencies that may relate to their differential ENTO sensitivity.

Entospletinib potently inhibits in vivo acute lymphoblastic leukemia proliferation with enhanced efficacy in combination with chemotherapy

We then investigated the extent to which ENTO could inhibit in vivo leukemia proliferation in ALL PDX models when administered as monotherapy or in combination with vincristine (VCR) chemotherapy. We observed that combined ENTO and VCR treatment resulted in superior inhibition of ALL proliferation in a KMT2A-MLLT3 (ALL3103) model and a KMT2A-MLLT1 (ALL135MR) model (both RAS wild-type) than was observed with single-agent ENTO or VCR (P<0.001 and P<0.05, respectively) (Figure 5A). Superior leukemic cell depletion with ENTO and VCR combination was confirmed by quantitative CD19 IHC in harvested murine spleens and bone marrow (see Online Supplementary Figure S10 for representative ALL3103 data). Conversely, drug treatment of two RAS-mutant KMT2A-R ALL PDX models (Figure 5B) showed marked vincristine-induction reduction of leukemic burden (ALL142MR, P<0.0001; ALL150MD, P<0.001) but no effects of ENTO monotherapy or additional treatment effect of combined ENTO and VCR. Evaluation of an adult RAS wild-type KMT2A-AFF1 ALL PDX model (ALL3115) showed significant treatment effects of ENTO alone and in combination with VCR (P<0.0001 for both) (Figure 5C), contrasting with effects observed in the RAS-mutant models. Taken together, these data indicate that RAS mutations in KMT2A-R subtypes may overcome or prevent potential anti-leukemia activity of ENTO.

We then explored treatment effects of ENTO in a control non-KMT2A-R ALL PDX model with t(1;19) resulting in TCF3-PBX1 fusion and a KRAS G12D mutation (ALL132GD), which we expected to be sensitive to ENTO given typical pre-BCR expression on this more mature B-ALL subtype 42,43 and confirmed by positive FC immunoglobulin µ-heavy chain staining on AALL132GD cells (data not shown). However, we saw no response to single-agent ENTO or in combination with VCR, further substantiating the potential impact of RAS mutations upon ENTO insensitivity (Figure 5D). Finally, we tested ENTO and VCR in two RAS wild-type non-KMT2A-R ALL PDX models (ALL185GD and ALL83GD) (Figure 5E). We observed sensitivity of model ALL185GD to ENTO monotherapy (P<0.05) and in combination with VCR (P<0.0001), although the latter effects did not differ from those of VCR monotherapy. Model ALL83GD was not sensitive to ENTO alone, but showed significant combinatorial treatment efficacy versus ENTO or VCR monotherapy (P<0.0001 and P<0.05, respectively). Interestingly, we discovered that the ALL185GD and ALL83GD non-KMT2A-R models have P2RY8-CRLF2 fusions with expected constitutive activation of JAK/STAT signaling (Figure 4B). Our group recently reported an essential role

Figure 2. Activity and dose optimization of entospletinib monotherapy in KMT2A-R acute lymphoblastic leukemia (ALL). (A) Viably cryopreserved harvested human KMT2A-R ALL cells from murine PDX spleens (model ALL3103 with KMT2A-MLLT3 fusion) demonstrated dose-dependent inhibition of colony formation in vitro in methylcellulose colony assays after ENTO treatment for 14 days. Samples were plated in triplicate in methylcellulose-based medium and grown in 10% leukocyte-conditioned medium with 25% FBS and 2% BSA. Data are displayed as mean ± SEM. (B) ALL3103 PDX mice were treated with vehicle (control) or ENTO chow at the specified concentrations for 4 weeks. Human CD45+ CD19+ ALL flow cytometric analysis of murine blood at weekly time points and (C) spleens at study endpoint demonstrated significant inhibition of ALL proliferation with ENTO treatment (mean ± SEM). No difference in ALL burden was observed in 0.03% versus 0.07% ENTO-treated animals. (D) Terminal blood was collected from animals after 4 weeks of continuous ENTO chow consumption and evaluated for entospletinib levels. Data from individual animals are plotted as median interquartile range. ns: not significant by t-test. (E) Terminal spleens from individual mice were harvested, viably cryopreserved, lysed, and evaluated for levels of pSYK, SYK, cMYC, pERK and β-actin by Simple Western. *P<0.05, **P<0.01, ****P<0.0001 as compared to control chow-fed mice by ANOVA with Tukey’s post-test.
of SFK signaling in CRLF2-rearranged Ph-like ALL with \textit{in vitro} and \textit{in vivo} sensitivity to the kinase inhibitor dasatinib\cite{44,45} and hypothesize that the observed ENTO sensitivity in our CRLF2-R infant ALL models could be due to a similar mechanism and signaling dependency.

**Superior preclinical activity of combined SYK and MEK inhibition in KMT2A-R acute lymphoblastic leukemia patient-derived xenograft models**

Given the surprising observed lack of ENTO activity in our RAS-mutant \textit{KMT2A-AFF1} infant ALL PDX models, we hypothesized that dual treatment with ENTO and a MEK inhibitor (MEKi) would have superior therapeutic effects. To test this prediction, we treated RAS-mutant (ALL142MR; infant) and RAS wild-type (ALL3113MR; adult) \textit{KMT2A-AFF1} ALL PDX models with ENTO, selumetinib (SEL), or both kinase inhibitors and quantified ALL cell counts in peripheral blood during treatment and in end-study spleens. As expected,\cite{40,46} single-agent SEL treatment of the RAS-mutant ALL142MR model appreciably decreased leukemia burden and augmented anti-ALL effects in combination with ENTO (Figure 6A). Despite its lack of RAS mutation, the ALL3113 model was surprisingly sensitive to SEL monotherapy\cite{41,48} and potent \textit{in vivo} activity with near-complete leukemia clearance was observed with dual ENTO and SEL treatment (Figure 6B). These \textit{in vivo} efficacy data in both RAS-mutant and wild-type models, and our additional demonstration of constitutive pERK levels and \textit{ex vivo} signaling inhibition in end-study spleens of both ALL142MR and ALL3113 models (Figure 6C), suggest that MEK inhibition may be a relevant therapeutic strategy for \textit{KMT2A} ALL irrespective of RAS mutation status and may augment SYK inhibitor effectiveness.

**Discussion**

SYK pathway activation plays a central role in the proliferation and survival of malignant B cells, implicating...
SYK as a potential therapeutic target. Preclinical studies have shown that SYK inhibition can attenuate the growth of B-ALL in vitro and in vivo regardless of pre-BCR expression or genetic subtype.26,29 Mohr et al. also recently reported that HOXA9/MEIS1-induced upregulation of SYK is a major driver of leukemogenesis in AML.25 Several early phase clinical trials are now testing the safety and potential efficacy of ENTO in combination with chemotherapy in adults with relapsed or refractory leukemias (clinicaltrials.gov identifiers: NCT02404220, NCT02343939, NCT03135028). Interim results from these studies have reported manageable adverse events and remarkable response rates, particularly in patients with KMT2A-R AML (clinicaltrials.gov identifier: NCT02343939).33

Figure 4. HOXA9 and MEIS1 expression signatures of KMT2A-R and non-KMT2A-R acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) specimens. (A) Splenic PDX samples were analyzed for expression of mRNA for HOXA9 and MEIS1 by NanoString, with human bone marrow mononuclear cells (BMMC) and KG-1 cell line as negative and positive controls, respectively. Increased MEIS1 and/or HOXA9 expression was seen in KMT2A-R ALL PDX models versus non-KMT2A-R (WT) models and generally clustered by genetic subtype. (B) Total and phosphorylated signal transduction proteins from murine splenic lysates were evaluated using Simple Western. Basal kinase signaling activation differed among KMT2A-R and non-KMT2A-R ALL samples and stratified by genetic subgroup (KMT2A-AFF1, KMT2A-MLLT1, KMT2A-MLLT3, and non-KMT2A-R). β-actin was used as a protein loading control.
Chemotherapy resistance and subsequent relapse remain a major cause of childhood cancer mortality, especially for infants with KMT2A-R B-ALL who have extremely poor EFS. In one study, Pieters et al. reported 3-fold higher risk of relapse or death in infants with KMT2A-R ALL (irrespective of KMT2A rearrangement subtype) versus those without KMT2A rearrangements. Outcomes for infants with the KMT2A-AFF1 subtype from t(4;11) are particularly poor, although differences in associated HOX family gene expression and presence or absence of reciprocal AFF1-KMT2A fusions may contribute to differential clinical outcomes, as shown recently by Agras-Doblas and Bueno et al. in a large analysis of infant ALL specimens from the European co-operative groups’ Interfant-99 and -06 trials and reviewed by Slany.

Several groups have hypothesized that addition of targeted inhibitors to frontline chemotherapy could decrease relapse risk and improve survival for infants with ALL, as has been shown with tyrosine kinase inhibitors (TKI) for patients with BCR-ABL1-rearranged (Ph+) ALL. Unfortunately, addition of the FMS-like tyrosine kinase 3

Figure 5. Entospletinib potently inhibits in vivo acute lymphoblastic leukemia (ALL) proliferation with enhanced efficacy in combination with chemotherapy.

Animals engrafted with KMT2A-R (ALL3103, ALL135MR, ALL142MR, ALL3113) or non-KMT2A-R (ALL132GD, ALL185GD, ALL83GD) ALL were treated with control chow, 0.05% ENTO chow, 0.1 mg/kg vincristine (VCR) IP weekly, or both ENTO and VCR. Human CD45+CD19+ ALL cells were quantified by flow cytometry in end-of-study murine spleens and peripheral blood. (A) Combined ENTO+VCR significantly inhibited leukemia proliferation with enhanced activity compared to ENTO and/or VCR monotherapies in KMT2A-R PDX models without RAS mutations. (B) Conversely, potent VCR effects were observed in KMT2A-R ALL PDX models with NRAS or KRAS mutations without additional activity of combination treatment. (C) A KMT2A-R RAS wild-type ALL PDX model was sensitive to ENTO and not to VCR. (D) No treatment effects of ENTO or VCR were observed in a non-KMT2A-R RAS mutant ALL PDX model, while single-agent activity of VCR and/or ENTO and enhanced effects of combination treatment were detected in (E) non-KMT2A-R RAS wild-type PDX control models with other ALL-associated translocations. Data were analyzed by one-way ANOVA with Tukey’s post-test for multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
SYK inhibition for infant ALL

Inhibitor (FLT3i) lestaurtinib did not improve EFS for infants with KMT2A-R B-ALL (which usually have high FLT3 receptor [CD135] surface expression) in the COG trial AALL0681, which was likely in part attributable to insufficient PD target inhibition observed by correlative plasma inhibitory activity (PIA) assays.50,51 Similarly, no appreciable efficacy of the FLT3i quizartinib (AC220) was observed in children with relapsed KMT2A-R ALL in the TACL2009-004 phase I clinical trial (clinicaltrials.gov identifier: NCT011411267), although complete responses occurred in 3 of 7 patients with relapsed KMT2A-R AML with 94-100% FLT3 inhibition by PIA assay seen in all studied patients.52 Despite promising preclinical data,53,54 clinical activity of DOT1L inhibitors (e.g., pinemetostat [EPZ-5676]) targeting the KMT2A complex was similarly disappointing in children with relapsed KMT2A-R leukemias (clinicaltrials.gov identifier: NCT02141828),55 again potentially due to insufficient achievable drug levels considered necessary for response. Menin inhibitors targeting the KMT2A complex have shown exciting preclinical activity and may have superior pharmacologic properties, but have not yet entered clinical testing. Finally, current or planned trials are assessing the potential activity of 5-azacytidine priming (COG AALL15P1; clinicaltrials.gov identifier: NCT02828358) or blinatumomab specifically in infants with KMT2A-R ALL; however, results of these approaches are not yet known.

Our current study sought to define the potential activity of the selective SYK inhibitor ENTO specifically in preclinical infant KMT2A-R PDX models. Our demonstration of in vitro and in vivo anti-leukemia activity of ENTO with enhanced effects in combination with VCR or dexamethasone (critical and commonly-used anti-ALL chemotherapy agents) provides a rationale for further evaluation of SYK inhibition as a therapeutic strategy for this high-risk leukemia subtype. Interestingly, we observed minimal activity of ENTO alone or with VCR in KMT2A-R leukemias harboring concomitant RAS muta-
tions. This observation extended to a control non-KMT2A-R infant ALL model with a TCF3-PBX1 fusion from t(1;19), which had a concomitant KRAS mutation and was also insensitive to ENTO. Prior studies have shown that RAS mutations occur significantly more frequently in infants with B-ALL, particularly in those with the most common KMT2A-AFF1 subtype. Data do not agree as to whether ALL-associated RAS mutations confer higher relapse risk and inferior overall survival.4,34,35,36

The potential role of RAS mutations in conferring insensitivity to SYK inhibition in ALL was further extended by evaluation of ENTO in combination with the clinically-available MEK inhibitor selumetinib in two KMT2A-R ALL PDX models. As predicted,4,37 we observed significant inhibition of leukemia proliferation with SEL treatment of a RAS-mutant KMT2A-AFF1 infant ALL model with superior activity of ENTO and SEL combination. However, SEL monotherapy and combined SEL/ENTO therapy was also quite efficacious in a RAS wild-type KMT2A-AFF1 adult ALL model with high basal pERK levels. These data contrast somewhat with earlier preclinical data from Irving et al. demonstrating preferential activity of SEL (monotherapy or in combination with dexamethasone) in RAS-mutant ALL,40,41 an approach now under clinical investigation in children with relapsed/refractory RAS-mutant ALL via the SelDuEx phase I/II trial (clinicaltrials.gov identifier: NCT03705507), but are concordant with data from Kerstjens et al. reporting preclinical MEK inhibitor activity in both RAS-mutant and wild-type ALL.42 Cremer et al. also recently reported that MAPK pathway activation is a major mechanism of entospletinib or fostamatinib resistance in AML and can be overcome with dual SYK and MEK inhibition.43

In summary, our studies show constitutive activation of SYK and associated kinase signaling in preclinical infant KMT2A-R and childhood Ph-like ALL PDX models. We report potent in vitro and in vivo effects of selective SYK inhibition with enhanced activity of chemotherapy in non-RAS-mutant KMT2A-R ALL models. We also observed combinatorial activity of ENTO with the MEK inhibitor selumetinib in two KMT2A-R ALL PDX models with RAS mutation or pathway activation. Our findings warrant further evaluation of efficacy and toxicity of ENTO/SEL dual therapy in additional PDX models, potentially in combination with steroids or other traditional chemotherapy agents. Taken together, our preclinical studies demonstrate activity of ENTO in KMT2A-R ALL in combination with anti-ALL chemotherapy or MEK inhibition and suggest a potential for clinical evaluation of SYK inhibitor-based therapies in children and adults with these high-risk leukemias.

Disclosures

AV, MW, AS and ST are current or former employees of Gilead Sciences and have equity ownership in Gilead Sciences. SKT received research funding from Gilead Sciences. The remaining authors declare no relevant conflicts of interest.

Contributions

JPL and AV designed and performed research, analyzed data, and contributed to writing the manuscript; PAB contributed vital new reagents, and analyzed and interpreted data; LMN, AB, MW and AS performed research and analyzed data; ST and SKT oversaw the study, designed research, analyzed and interpreted data, and wrote the manuscript; SKT was responsible for revision of the manuscript; all authors approved the final version of the manuscript.

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