ARTICLE

# Combinatorial efficacy of entospletinib and chemotherapy in patient-derived xenograft models of infant acute lymphoblastic leukemia

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## ABSTRACT

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.

## Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is the most common childhood cancer and is characterized by recurrent somatic cytogenetic and molecular abnormalities. While modern risk-adapted chemotherapy regimens for children and adolescents/young adults (AYA) have achieved overall survival rates exceeding 90%,[1,2] optimal salvage therapy for the 10-15% of children and >60% of adults with B-ALL who relapse remains a major unmet medical need.[3-5]

Patients with B-ALL harboring rearrangements in lysine-specific methyltrans-

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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.[6-8] *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.[8,9] Children with *KMT2A*-rearranged (*KMT2A*-R) ALL have a poor prognosis with 5-year event-free survival (EFS) of 20-50% in infants[9-12] and approximately 58% in older children.[13] 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.[10,11] Adults with *KMT2A*-R ALL have similarly poor outcomes with <50% 5-year EFS.[14]

Wild-type *KMT2A* is required for normal hematopoiesis and post-natal hematopoietic cell maintenance.[15] Disruption of *KMT2A* via chromosomal translocation in acute lymphoid and myeloid leukemias was first described nearly three decades ago.[16,17] 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*.[18-20] 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*.[21] 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-AFF1* 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;q23) with *KMT2A-MLLT4* fusion (1%).[8,22-24]

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),[21,25] as well as constitutive activation of SYK signaling in several B-ALL subtypes.[2,26] 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).[27] 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.[28,29] *In vitro* and *in vivo* activity of SYK inhibition in preclinical B-ALL models has been previously established[26,30,31] 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)[32] 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.[33] 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=3) enrolled on the Children's Oncology Group (COG) trial AALL0631 were obtained via informed consent as previously described.[34] Additional specimens from an infant with relapsed *KMT2A*-R (n=1; ALL3103) 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 Il2rgtm1Wjl/SzJ* (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.[35-38] Additional established non-*KMT2A*-R ALL PDX models (primarily of the Philadelphia chromosome-like [Ph-like] subtype)[15,37-39] (*Online Supplementary Table S1*) were used as negative controls.

### Kinase inhibitors and chemotherapy

The selective SYK inhibitor entospletinib (ENTO)[32] 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*).[33] 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 cytom-

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

| ALL PDX model | COG USI | *KMT2A* status | Translocation | Disease status | Other genetic alterations |
|---|---|---|---|---|---|
| ALL185GD | PAVVRD | wild-type | *P2RY8-CRLF2*, *PAX5-AUTS2* | *De novo* | *JAK2* mut, *CDKN2A/B* del |
| ALL83GD | PAUFHC | wild-type | *P2RY8-CRLF2*, *PAX5-C20orf112* | *De novo* | *JAK2* del, *CDKN2A/B* del, *RTEL* del |
| ALL132GD | PAUXSA | wild-type | t(1;19) (q23;p13) with *TCF3-PBX1* | *De novo* | *KRAS* mut, *WHSC1* mut, gain *CCND3, MYB, ESR1* |
| ALL150MD | PAVEDG | *KMT2A-AFF1* | t(4;11) (q21;q23) | *De novo* | *KRAS* mut |
| ALL142MD | PAVBRV | *KMT2A-AFF1* | t(4;11) (q21;q23) | *De novo* | *NRAS* mut |
| ALL142MR | PAVBRV | *KMT2A-AFF1* | t(4;11) (q21;q23) | Relapse | *NRAS* mut, *IKZF1* del, cnLOH of chr22 |
| ALL3113MR | n/a | *KMT2A-AFF1* | t(4;11) (q21;q23) | *De novo* | *JAK2* mut, *TP53* 17p del, *IKZF1* 7p del |
| ALL3103MR | n/a | *KMT2A-MLLT3* | t(9;11) (p21;q23) | Relapse | None identified |
| ALL135MD | PAUYJT | *KMT2A-MLLT1* | t(11;19) (q23;p13.3) | *De novo* | None identified |
| ALL135MR | PAUYJT | *KMT2A-MLLT1* | t(11;19) (q23;p13.3) | Relapse | None identified |
| ALL26MD | PASHFM | *KMT2A-MLLT1* | t(11;19) (q23;p13.3) | *De novo* | None identified |
| ALL26MR | PASHFM | *KMT2A-MLLT1* | t(11;19) (q23;p13.3) | Relapse | Partial 10q del, including *PTEN* |

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

etry signaling analyses of human B-ALL cell lines and PDX model cells treated with vehicle, kinase inhibitors, or chemotherapy (*in vitro* or *in vivo*) are detailed in the *Online Supplementary Methods* with data shown in *Online Supplementary Figures S1-S6*.

### *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 pharmacodynamics [PD] studies) or up to 28 days (treatment efficacy studies) as described.[37,38] 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[40] 5 days/week as (ALL135MR and ALL3113) or dexamethasone 1 mg/kg PO once daily 5 days/week (ALL3113, ALL83GD) as monotherapy or in combination with ENTO. Further details about *in vivo* drug testing in ALL PDX models and conduction 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 *HOXA9* and *MEIS1* expression,[25] and early reports of clinical responses in adults with relapsed *KMT2A*-R leukemias treated with entospletinib[42,43] 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,[26,29] including RAS/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.

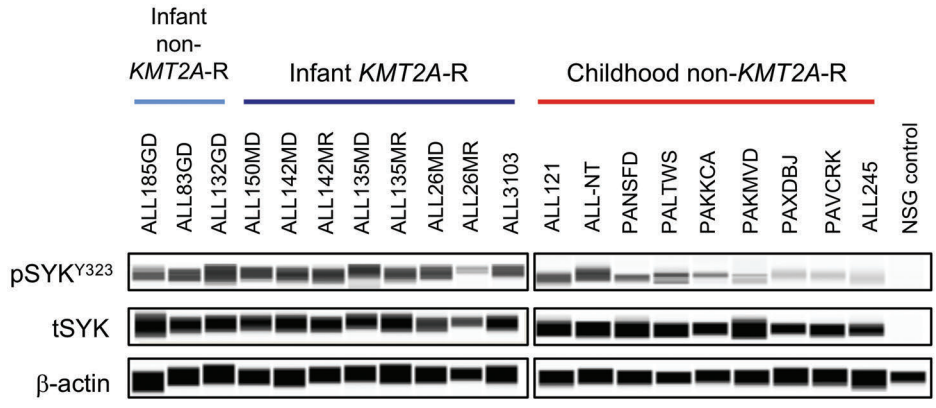


**Figure 1. Constitutive SYK signaling occurs in infant acute lymphoblastic leukemia (ALL).** Simple Western analysis of splenic lysates from human ALL patient-derived xenograft (PDX) models demonstrated high basal phosphorylated SYK (pSYK) levels in the majority of infant non-*KMT2A*-rearranged (R) (light blue) and *KMT2A*-R (dark blue) ALL specimens. pSYK levels were lower in most childhood non-*KMT2A*-R ALL specimens (red) and absent in splenic tissue from non-leukemia-injected NSG mice (gray). Total SYK levels were similar across all models. ALL PDX model names are specified above corresponding Simple Western data.

We then assessed the ability of ENTO to inhibit leukemia proliferation *in vivo* in ALL3103 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 ALL3103 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, $pAKT^{S473}$, 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 $NRAS^{G12D}$ 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.[1,21]

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

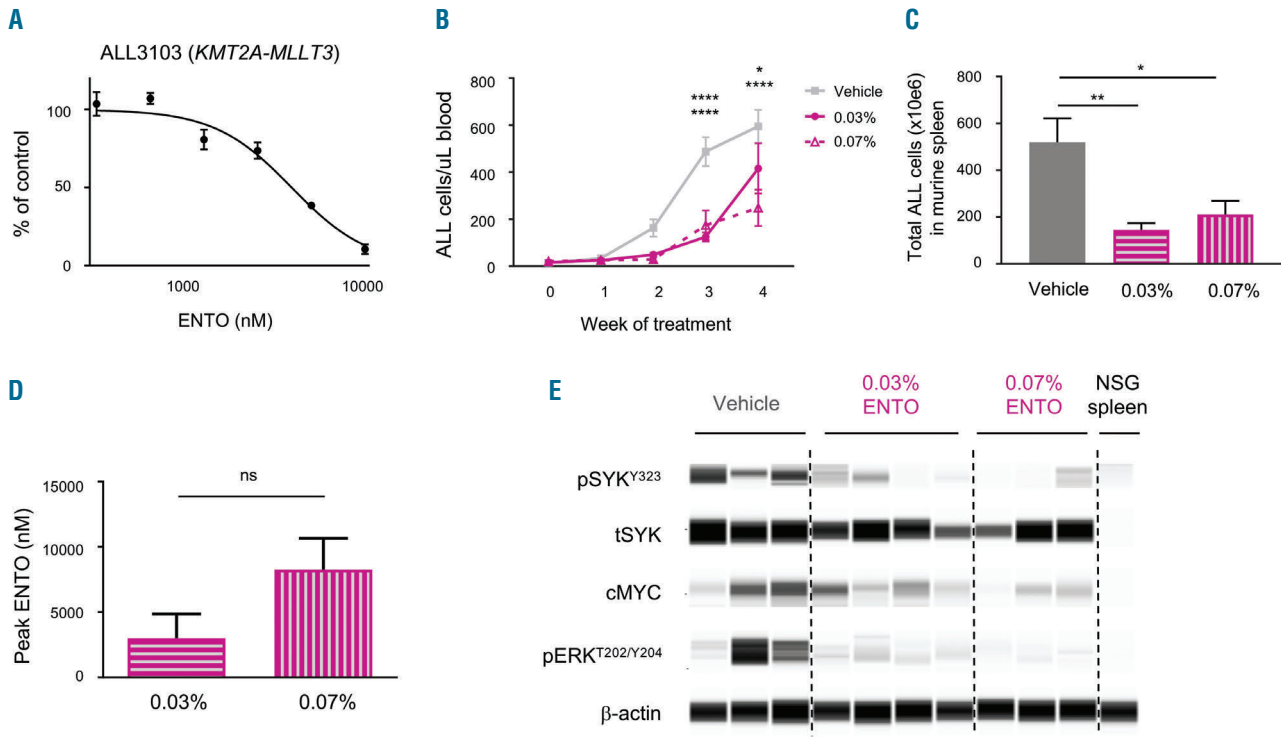


**Figure 2. Activity and dose optimization of entospletinib monotherapy in *KMT2A*-R acute lymphoblastic leukemia (ALL).** (A) Viably cryopreserved harvested human *KMT2A*-R ALL cells from murine PDX spleens (model ALL3103 with *KMT2A-MLLT3* fusion) demonstrated dose-dependent inhibition of colony formation *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.

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 (ALL3113) 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

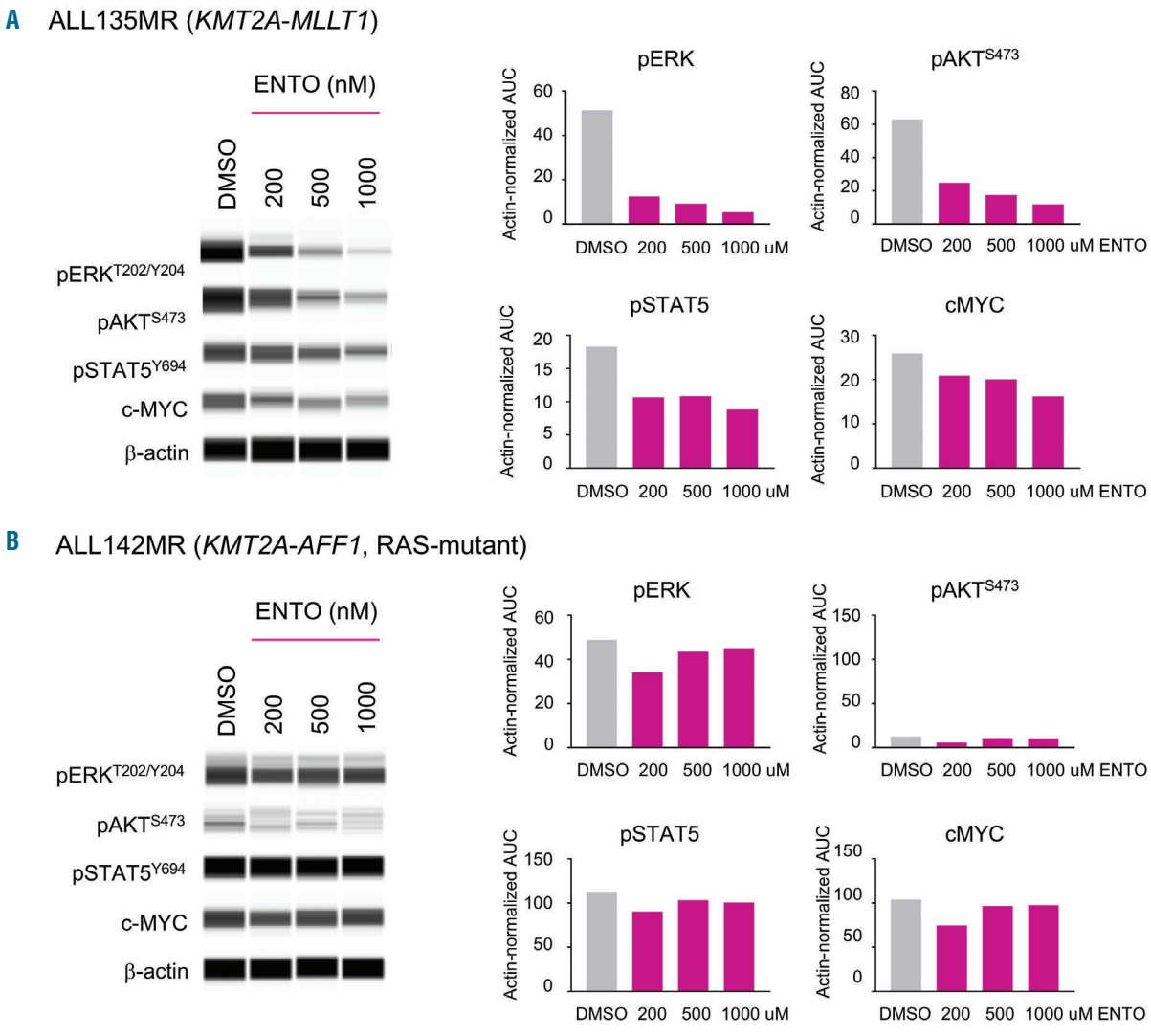


**Figure 3. *In vitro* activity of entospletinib in *KMT2A*-R acute lymphoblastic leukemia (ALL).** Viably cryopreserved KMT2A-R ALL PDX cells were exposed *in vitro* to 0.1% DMSO (vehicle control) or increasing concentrations of entospletinib (200 nM, 500 nM, 1 uM) for 2 hours, then lysed and analyzed by Simple Western. Additional untreated (baseline) cells were lysed immediately following sample thaw. (A) Dose-dependent inhibition of the specified phosphoproteins was observed with ENTO in the ALL135MR PDX model (*KMT2A-MLLT1*, RAS wild-type), while (B) no treatment effect was seen in the 142MR PDX model (*KMT2A-AFF1*, *NRAS*-mutant).

of SFK signaling in *CRLF2*-rearranged Ph-like ALL with *in vitro* and *in vivo* sensitivity to the kinase inhibitor dasatinib[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 *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) *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,[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[41,48] and potent *in vivo* activity with near-complete leukemia clearance was observed with dual ENTO and SEL treatment (Figure 6B). These *in vivo* efficacy data in both RAS-mutant and wild-type models, and our additional demonstration of constitutive pERK levels and *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 *KMT2A*-R 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]

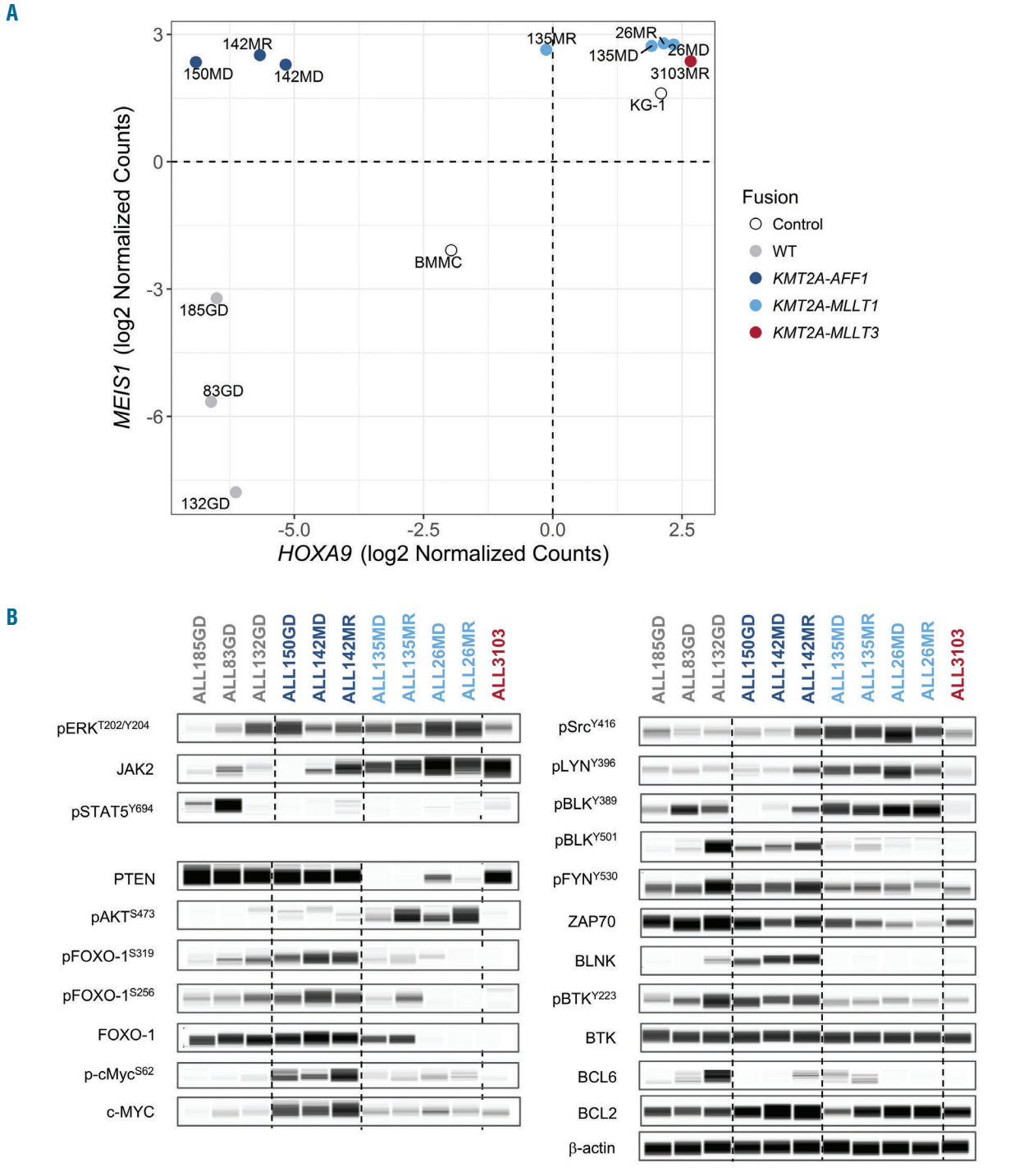


**Figure 4. *HOXA9* and *MEIS1* expression signatures of *KMT2A*-R and non-*KMT2A*-R acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) specimens.** (A) Splenic PDX samples were analyzed for expression of mRNA for *HOXA9* and *MEIS1* by NanoString, with human bone marrow mononuclear cells (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.[10] 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[47-49] and reviewed by Slany.[20]

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

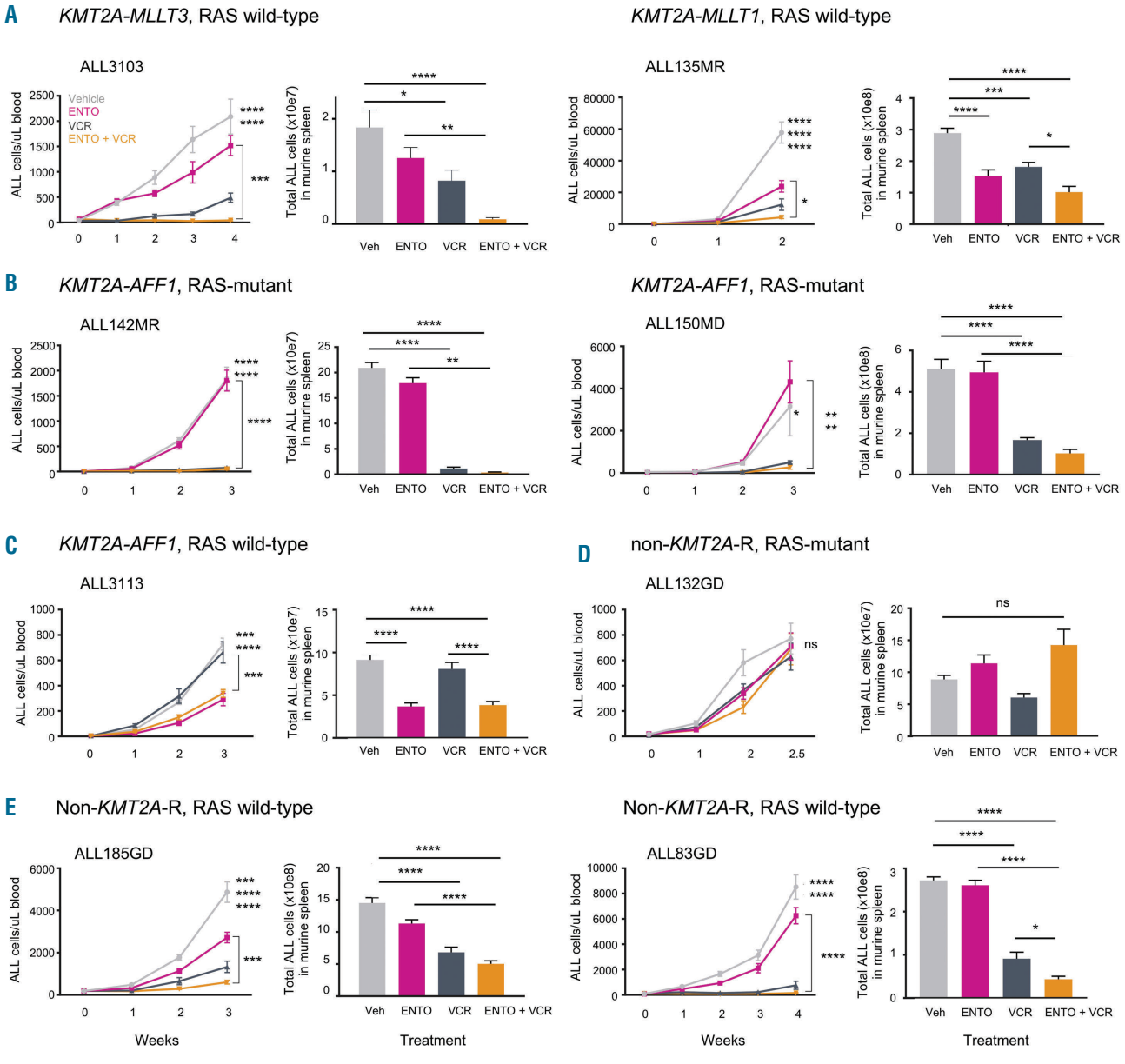


**Figure 5. Entospletinib potently inhibits *in vivo* acute lymphoblastic leukemia (ALL) proliferation with enhanced efficacy in combination with chemotherapy.** Animals engrafted with *KMT2A*-R (ALL3103, ALL135MR, ALL142MR, ALL150MD, ALL3113) or non-*KMT2A*-R (ALL132GD, ALL185GD, ALL83GD) ALL were treated 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 *KRAS*-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.

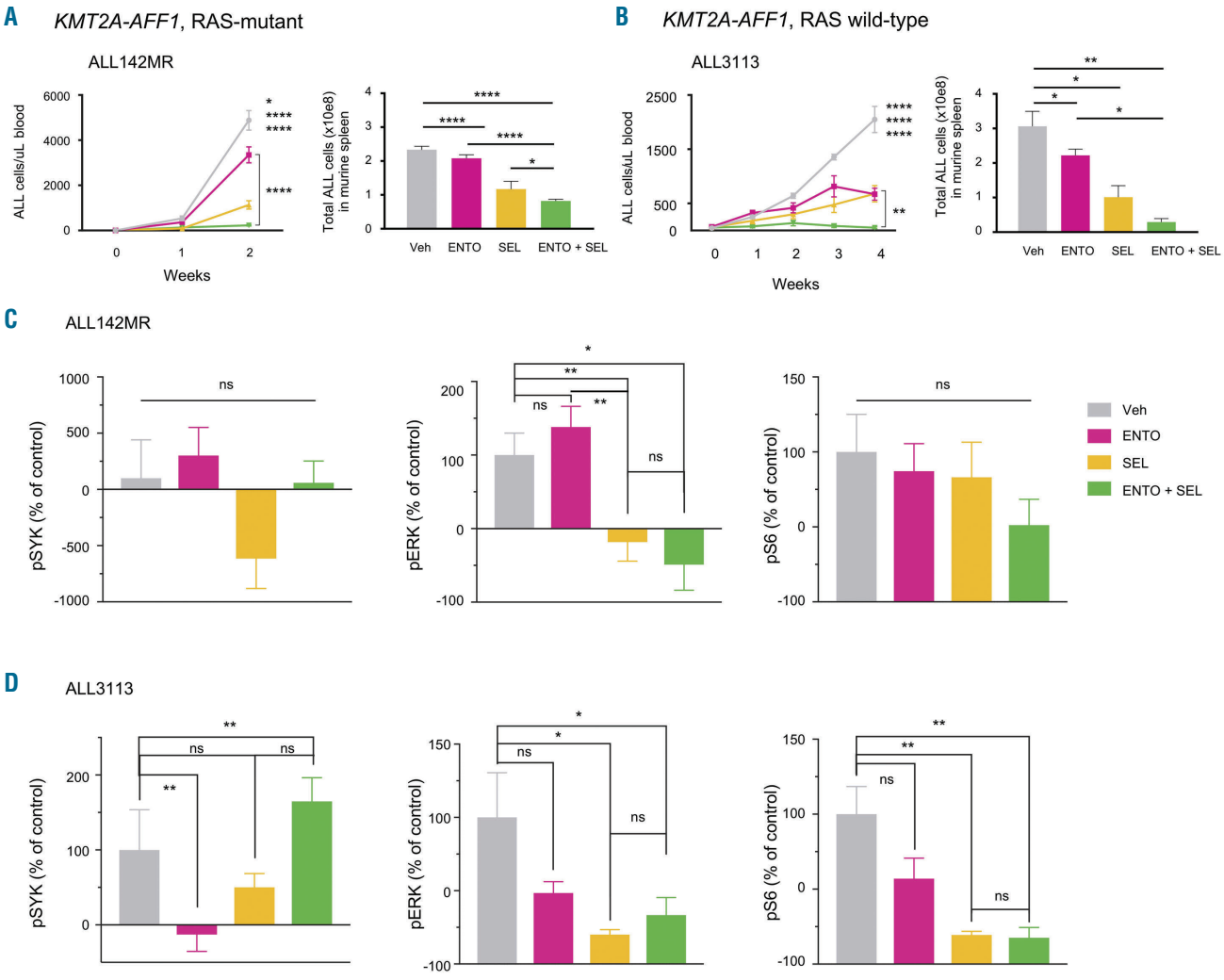


**Figure 6. Superior preclinical activity of entospletinib and selumetinib in *KMT2A*-R acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) models.** (A) PDX models 142MR (*KMT2A-AFF1*, *NRAS*-mutant) and (B) ALL3113 (*KMT2A-AFF1*, RAS wild-type) were treated with vehicle control chow, 0.05% ENTO chow, 100 mg/kg selumetinib (SEL) via oral gavage twice daily 5 days/week, or both ENTO and SEL for 2 or 4 weeks. Human CD45$^+$ CD19$^+$ ALL cells were quantified by flow cytometry in peripheral blood and end-study murine spleens. Enhanced anti-leukemia efficacy was observed in both models with combined ENTO and SEL treatment *versus* ENTO or SEL alone, as measured by one-way ANOVA with post Tukey's post-test for multiple comparisons. *$P$<0.05, **$P$<0.01, ****$P$<0.0001. (C) *Ex vivo* phosphoflow cytometry analysis of gated human CD19$^+$ CD45$^+$ ALL cells in end-study murine spleens after 2 weeks (ALL142MR) or 4 weeks (ALL3113) of ENTO and/or SEL treatment demonstrate inhibition of pSYK, pERK, and/or pS6 *versus* vehicle control (gray). ns: not significant, *$P$<0.05, **$P$<0.01 by one-way ANOVA with post Tukey's post-test for multiple comparisons.

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 AALL0631, 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 *FLT3*-mutant 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[56] 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 ALL 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.[8,24,57,58]

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,[40,59] 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,[40,46] an approach now under clinical investigation in children with relapsed/refractory RAS-mutant ALL via the SeluDex 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.[59] 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.[60]

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 with 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

*AY, 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 AY 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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