Resistance Mechanisms to SYK Inhibition in Acute Myeloid Leukemia

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ABSTRACT

Spleen tyrosine kinase (SYK) is a nonmutated therapeutic target in acute myeloid leukemia (AML). Attempts to exploit SYK therapeutically in AML have shown promising results in combination with chemotherapy, likely reflecting induced mechanisms of resistance to single-agent treatment in vivo. We conducted a genome-scale open reading frame (ORF) resistance screen and identified activation of the RAS–MAPK–ERK pathway as one major mechanism of resistance to SYK inhibitors. This finding was validated in AML cell lines with innate and acquired resistance to SYK inhibitors. Furthermore, patients with AML with select mutations activating these pathways displayed early resistance to SYK inhibition. To circumvent SYK inhibitor therapy resistance in AML, we demonstrate that a MEK and SYK inhibitor combination is synergistic in vitro and in vivo. Our data provide justification for use of ORF screening to identify resistance mechanisms to kinase inhibitor therapy in AML lacking distinct mutations and to direct novel combination-based strategies to abrogate these.

SIGNIFICANCE: The integration of functional genomic screening with the study of mechanisms of intrinsic and acquired resistance in model systems and human patients identified resistance to SYK inhibitors through MAPK signaling in AML. The dual targeting of SYK and the MAPK pathway offers a combinatorial strategy to overcome this resistance.
INTRODUCTION

After a frustrating decade of limited progress in the treatment of patients with acute myeloid leukemia (AML), 2017–2018 was a remarkable turning point. The FDA approved for marketing new agents for patients with this disease: liposomal daunorubicin/cytarabine, enasidenib, ivosidenib, gemtuzumab ozogamicin, venetoclax, midostaurin, and gilteritinib. A next wave of drugs is coming down the pike targeting genes not mutated in AML, with several showing evidence of early clinical activity. A challenge that lies ahead is to leverage these new targeted agents toward maximal clinical efficacy.

One targeted approach for patients with AML recently showing promising signs of activity is the inhibition of spleen tyrosine kinase (SYK). SYK is a cytoplasmic tyrosine kinase best known for its role in B-cell development but also characterized to play a role in myeloid signaling more broadly (1–3). Multiple lines of preclinical evidence suggest the therapeutic targeting of SYK in AML. In rare instances, SYK is hyperactivated in myeloid malignancies through gene fusions, such as TEL–SYK (4, 5), whereas in other instances, it is activated through integrin and Fc receptor signaling (3, 6). Genetic suppression, as well as chemical perturbation of SYK activity, resulted in impaired growth of AML cells in vitro and in mouse models of AML and induced differentiation in some AML contexts (6, 7). Adding further credence to an important role for SYK in AML, two independent studies reported high levels of SYK phosphorylation in AML bone marrow specimens as a poor prognostic marker relative to therapeutic outcome (8, 9). Finally, candidate biomarkers of response to SYK inhibitors have included FLT3 mutations and high levels of HOXA9 and MEIS1 expression (9–11). Notably, SYK inhibitors have been shown to be active in the high-risk FLT3-mutated AML subset (10–13) where SYK was demonstrated to physically interact with and activate FLT3. In addition, in a recent report from the Beat AML program, mutations in NPM1 alone or in combination with FLT3-ITD or DNMT3A mutations were also predictive of response to SYK inhibition in primary patient samples treated in vitro (11).
Two orally bioavailable SYK inhibitors, entospletinib and TAK-659, have entered clinical trials for patients with AML, with both studies demonstrating early evidence of response, including a modest number of complete responses with single-agent treatment (14–16). More strikingly, in one study combining the SYK inhibitor entospletinib with standard chemotherapy (cytarabine and daunorubicin), patients with FLT3 mutations, MLL rearrangements, and NPM1 mutations had a higher than predicted complete response rate compared with historical controls (15). Intriguingly, NPM1-mutated AML is another subset reported to have high expression of HOXA9 and MEIS1, and in further support of the preclinical studies, HOXA9 and MEIS1 expression were associated with a trend toward higher incidence of complete remission in this clinical trial (17).

Although these early clinical trial results are encouraging, targeted therapy is typically associated with the emergence of resistance, and combination therapy is almost always needed for a durable therapeutic response (18). The most frequent mechanism of acquired resistance is the development of, or selection for, secondary mutations in the drug target (19, 20). Patients can, however, also acquire mutations in genes that encode upstream or downstream effectors of the targeted signaling pathway, leading to its reactivation. Finally, different signaling hubs can be activated to compensate for inhibition of the drug target (21). For example, small-molecule inhibitors of oncogenic BRAFV600E in colon cancer are circumvented through the activation of feedback loops that engage EGFR (22), which leads to the reactivation of the MAPK and PI3K pathways. In the case of BRAF, to optimize clinical efficacy of this targeted therapy, multiple preclinical studies with genome-wide screens were conducted to decipher resistance mechanisms. Mechanisms elucidated in those studies precisely predicted response to treatment in human patients and paved the way for new drug combinations entering the clinic (23).

Resistance mechanisms to SYK inhibition in AML have not been explored, and SYK is only rarely mutated in human disease, including in AML. For genes such as SYK, which do not typically have mutations but are constitutively active in a subset of patients, the mechanism of resistance is poorly understood. Here, we identified resistance mechanisms to SYK inhibition by using an integrated proteogenomic approach, including the study of primary patient samples as part of a clinical trial. We show that SYK inhibition leads to the downregulation of central key signaling nodes, such as the mTOR–AKT, JAK–STAT, and RAS–MAPK–ERK pathways, but that resistance mechanisms to SYK inhibition are mainly conveyed by reactivation of the RAS–MAPK–ERK signaling pathway and can be overcome by combining a MEK inhibitor with a SYK inhibitor.

RESULTS
Entospletinib Inhibits Downstream Targets of SYK

Entospletinib is an orally available, selective inhibitor of SYK (21), which was investigated in clinical trials in chronic lymphoid leukemia and non-Hodgkin lymphoma (21, 22), as well as recently in AML (14, 15). We first characterized response to entospletinib in a panel of AML cell lines and identified MV4-11, MOLM13, and the derivative cell line MOLM14 as among the most sensitive cell lines (Supplementary Fig. S1A and S1B; Supplementary Table S1). The IC50 values for these two cell lines match the plasma concentration of entospletinib in patients given a 400 mg twice a day dosing schedule (15, 24).

Next, we determined the effects of SYK inhibition with entospletinib on intracellular signaling pathways by combining stable isotope labeling by amino acids in cell culture (SILAC) and mass spectrometry–based phosphoproteomics. For this purpose, MV4-11 and MOLM13 cells were cultured for 1 hour with entospletinib at their respective IC50 concentrations, lysed, and phosphorylated tyrosine residues (pYome) enriched prior to mass spectrometry analysis. This quantitative phosphoproteome analysis allowed the quantification of up to 655 class I p-sites (localization probability >75%) in two replicate measurements in MV4-11 (Fig. 1A; Supplementary Table S2) and 522 p-sites in MOLM13 cells (Supplementary Fig. S1C; Supplementary Table S3).

In both cell lines, we detected reduced tyrosine phosphorylation for known effectors of SYK, such as STAT5 and CBL (ref. 6, 25; Fig. 1A; Supplementary Fig. S1C), consistent with on-target activity of entospletinib for SYK. We also detected reduced tyrosine phosphorylation of members of the RAS–MAPK–ERK signaling pathway, such as the downstream effectors MAPK3 (ERK1) and MAPK1 (ERK2), as well as the upstream mediators GAB2 and SHP2, consistent with prior reports that SYK inhibition negatively regulates RAS–MAPK–ERK signaling in AML (25). Concentration-dependent reductions in phosphorylation of SYK, STAT5, SHP2, and ERK1/2, were confirmed by immunoblotting in both MV4-11 (Fig. 1B) and MOLM14 cells (Supplementary Fig. S1D). When we investigated the corresponding genes from our quantitative phosphoproteome study with gene set enrichment analysis (GSEA), we detected a strong correlation between entospletinib treatment and the downregulation of different gene set categories including RAS–MAPK–ERK signaling, growth factor signaling, and chemokine/chemokine receptor signaling (Supplementary Fig. S1E and S1F).

In parallel, we analyzed the effects of SYK inhibition by entospletinib on genome-wide expression. MV4-11 cells were treated with vehicle or the IC50 concentration of entospletinib, and gene expression was profiled by RNA sequencing (RNA-seq). Data were analyzed with GSEA. We observed a strong correlation between entospletinib treatment and a previously published SYK-directed shRNA knockdown signature generated in AML cell lines [Fig. 1C, top; normalized enrichment score (NES) = −4.1, FDR < 0.01, P < 0.01; ref. 10] supporting the on-target activity of entospletinib against SYK in AML. Moreover, we observed a strong correlation with mTOR signaling inhibition (NES = −2.7, FDR < 0.01, P < 0.01; Fig. 1C, bottom), as described previously for other SYK inhibitors (25).

To further validate the antileukemic efficacy of entospletinib, we studied its activity in three different patient-derived xenograft (PDX) models of AML. These models had distinct AML-specific mutations (PDX #1: MLL-AF9; PDX #2: MLL-AF4; PDX #3: FLT3-ITD/NPM1). Treatment with entospletinib was initiated after confirming engraftment of transplanted cells as evidenced by human CD45 (hCD45)–positive cells in the peripheral blood by flow cytometry. Mice were sacrificed after 14 days of treatment and leukemia burden evaluated by detection of hCD45-positive cells. We observed...
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Figure 1. Characterization of effects of entospletinib in AML in vitro and in vivo. A, Intensities of peptide peaks versus mean log2 normalized (entospletinib/DMSO) ratios for p-sites identified by a mass-spectrometric pYome analysis in one out of two representative replicates. Red and black dots indicate p-sites downregulated in entospletinib/DMSO treated or upregulated, respectively, in MV4-11 cells (absolute mean log2 ratio entospletinib/DMSO ≥ 0.5, P ≤ 0.05, t-test with Welch correction); ns, not significant. Selected p-sites are labeled. B, Validation of selected differential tyrosine phosphorylation events by immunoblotting in MV4-11. Vinculin is used as a loading control. C, GSEA demonstrating enrichment of genes downregulated by entospletinib versus DMSO in MV4-11 cells. D, Percentage of hCD45-positive cells/live bone marrow cells after 14 days of entospletinib or vehicle treatment, respectively, in NSG mice injected with three different AML PDX models (*, P ≤ 0.05; ***, P ≤ 0.001, Mann-Whitney test). E, Survival study in NSG mice, injected with PDX #1 (left) and PDX #3 (right), randomly assigned to entospletinib or vehicle treatment, n = 8 mice per arm (P ≤ 0.0001, Mantel-Cox test).
a significant reduction in leukemia burden in all three PDX models (Fig. 1D; Supplementary Fig. S1G). In mice injected with PDX #1, we detected 82.34% (±3.059, n = 5) hCD45-positive bone marrow cells in the vehicle-treated group, in comparison with 65.38% (±4.277, n = 5) in the entospletinib-treated group. Mice injected with PDX #2 had 76.46% (±0.575, n = 5) hCD45-positive cells in the vehicle group versus only 50.78% (±2.419, n = 5) in the entospletinib-treated group, and PDX #3 had 20.9% (±1.427, n = 5) hCD45-positive cells in the bone marrow in the vehicle-treated group and 4.618% (±0.7602, n = 5) in the entospletinib-treated group. We evaluated for effects on survival in PDX #1 and PDX #3 where we observed a significant survival advantage for mice treated with entospletinib in comparison with vehicle-treated mice (PDX #1, P < 0.0001, n = 8; Fig. 1E, left; and PDX #3, P < 0.0001; n = 8; Fig. 1E, right). Taken together, we validated the efficacy of entospletinib in AML cell line and mouse models and defined the signaling pathways regulated by SYK in AML.

**Genome-Scale Lentiviral ORF Screen Identifies Pathways That Confer Resistance to Entospletinib in AML**

To study mechanisms of resistance that may evolve during entospletinib treatment, we performed a genome-scale, lentiviral open reading frame (ORF) screen in MV4-11 and MOLM14 cells. Cells were infected with a pooled lentiviral ORF library containing 17,255 barcoded ORFs that resulted in the overexpression of 10,315 distinct human genes with at least 99% nucleotide and protein match (26, 27). Next, cells were selected using puromycin, passed for three population doublings, and an early time point (ETP) was harvested (Fig. 2A). ORF-expressing cells were passaged for 21 days in the presence of 3 μmol/L (IC₅₀) entospletinib or a DMSO control (Supplementary Fig. S2A and S2B), genomic DNA (gDNA) isolated, and barcodes amplified and sequenced as described previously (28) on a HiSeq2000 (Illumina). The read counts were normalized to reads per million and then log₂ transformed. The log₂ fold change (FC) of each ORF was determined relative to the initial time point for each biological replicate (Fig. 2B and C). ORF representation for entospletinib treatment was strongly correlated between the MV4-11 and MOLM14 cell lines (Pearson R = 0.61, P < 2.2e-16; Fig. 2D) and between replicates in each cell line (Pearson R = 0.74, P < 2.2e-16 for MV4-11 and Pearson R = 0.60, P < 2.2e-16 for MOLM14; Supplementary Fig. S2C and S2D). ORFs were considered to be hits if they displayed z-scores (SDs from the mean) for log₂(FC) expression ≥ 2.0 with entospletinib versus ETP. There were 78 ORF hits in the MV4-11 cell line, corresponding to 62 genes (Fig. 2B and E; Supplementary Table S4) and 203 ORF hits corresponding to 168 genes in the MOLM14 cell line (Fig. 2C and E; Supplementary Table S5). After intersecting the top-scoring ORF hits in both cell lines, 24 genes were found to be in common (Fig. 2D and E; Supplementary Table S6). Importantly, top hits were specific to the drugs because they did not score in the DMSO control arm as promoting growth in the MV4-11 and MOLM14 cell lines. Genes that conferred resistance to entospletinib were significantly enriched for three major groups: growth factor receptors (e.g., EGFR, PDGFRα, and PDGFRβ), chemokine/chemokine receptors (e.g., CSF2, CSF3, and IL3), and RAS–MAPK–ERK signaling. Signaling via growth factor receptors, as well as signaling mediated by IL3 and GM-CSF, occurs at least in part via the RAS signaling pathway (29). Data from the ORF screen suggested that overexpression of wild-type RAS ORFs (HRAS and KRAS), as well as pathway activators (CRKL) and downstream effectors (e.g., RAF1, MAPK1, and MAPK3; Fig. 2D; Supplementary Tables S4–S6) conferred resistance to entospletinib in both screened AML cell lines. This genome-scale unbiased screen thus provided a strong rationale to further validate RAS as a driver of SYK inhibitor resistance.

RAS proteins, consisting of KRAS, NRAS, and H-RAS, promote growth-related signals from activated cell-surface receptors, as well as other kinases. RAS mutations are common in a variety of different cancer types, including myeloid malignancies, and result in constitutive activation of the RAS protein, which is held in the active GTP-bound state (30). In AML, approximately 15% of adult and up to 30% of pediatric patients harbor a hotspot mutation in the NRAS or KRAS genes, whereas mutations in H-RAS almost never occur in these patients (31–33). Therefore, further analysis focused on the RAS–MAPK–ERK signaling pathway, and specifically NRAS/KRAS, due to the frequency of RAS mutations in AML and their biological relevance in this disease.

We next overexpressed NRAS and KRAS ORFs in MV4-11 and MOLM14 cells and confirmed activation of the RAS–MAPK–ERK signaling pathway by detecting upregulation of phospho-ERK1/2 (Fig. 3A and B). We then treated these stably expressing cells with either DMSO or entospletinib and determined that overexpression of wild-type NRAS or KRAS promoted resistance to entospletinib-mediated cell death (Fig. 3C and D), confirming the ORF screen data.

To confirm our findings in another in vitro system, we infected primary MLL-APF9 rearranged murine bone marrow cells with retrovirus that contained an empty vector or mutated NRASG12D. After confirming expression of mutated NRASG12D by immunoblotting using a mutant-specific antibody, as well as upregulation of phospho-ERK1/2 (Fig. 3E), we tested the sensitivity to entospletinib and a structurally distinct SYK inhibitor, PRT062607 (34). In line with our previous results, the NRASG12D-expressing cells (IC₅₀ = 2.2 μmol/L) were 34-fold more resistant to entospletinib treatment than the control cells (IC₅₀ = 0.064 μmol/L; Fig. 3F) and 7-fold more resistant to the SYK inhibitor PRT062607 (Supplementary Fig. S3A).

**Hyperactivation of the RAS–MAPK Signaling Pathway Confers Innate Resistance to SYK Inhibition**

Next, we explored whether activation of the RAS–MAPK–ERK signaling pathway, through mutations in NRAS or KRAS, confers innate resistance to SYK inhibition. Indeed, when we exposed a panel of 12 different AML cell lines to entospletinib, we observed a difference between sensitive RAS wild-type cell lines (MV4-11, CMK-86, UCSD-AML1, MOLM14, MOLM13, and EOL-1) and cell lines that are RAS mutated (NOMO-1, TF-1, HL-60, THP-1, and NB4; ref. 35; Fig. 3G). The only wild-type RAS cell line that showed resistance to entospletinib treatment was U937. Interestingly, U937 harbors a PTPN11G600R mutation (36). Activating mutations of PTPN11 lead to a hyperactivation of the downstream RAS–MAPK–ERK signaling pathway (37). We observed a similar finding with analysis of a large dataset of primary AML samples treated...
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Figure 2. Genome-scale lentiviral ORFeome library screen identifies drivers of entospletinib resistance in AML. A, Schematic description of the genome-scale ORFeome library screen. B, Scatter plots presenting the z-scores for average log2 fold change ORF representation for entospletinib versus ETP (y-axis) and for DMSO versus ETP (x-axis) in MV4-11 (B) and MOLM14 (C) cells. Dotted gray lines at z-score = 2 on either axis demonstrating the ORF hits that are not scoring in the DMSO arm as promoting growth over time: DMSO versus ETP z-scores < 2 nominate genes not associated with enhanced growth in the DMSO, whereas entospletinib versus ETP z-scores ≥ 2 nominate genes associated with resistance to entospletinib. Genes with z-scores < 2 for DMSO and ≥ 2 for entospletinib were nominated as candidate genes conferring resistance and classified as significant ORFs. D, Scatter plots presenting the z-scores for average log2 fold change core ORF representation for entospletinib versus ETP in MOLM14 (y-axis) and for entospletinib versus ETP in MV4-11 (x-axis). E, Venn diagram presenting the 24 core ORF hits that scored in both cell lines MV4-11 and MOLM14 (P < 0.0001, Fisher exact test).

with various small-molecule inhibitors published as part of the Beat AML program (11). In this dataset, primary patient samples that harbored NRAS mutations were less sensitive to ex vivo entospletinib treatment (Fig. 3H).

**Acquired SYK Inhibitor Resistance Is Associated with RAS–MAPK Activation**

To extend our findings with regard to naturally acquired resistance mechanisms to SYK inhibitors, we generated resistant MV4-11 cells by gradually adapting cells to a maximum concentration of 5 μmol/L of entospletinib and treating them chronically over several months. We measured the IC50 of entospletinib in the resistant cell line model and detected a shift in sensitivity in comparison with the naïve cells (IC50 MV4-11: 0.5 μmol/L; IC50 MV4-11 resistant: 7.5 μmol/L; Fig. 4A). These cells were also cross-resistant to the SYK inhibitor PRT062607 (Fig. 4B).

To elucidate which signaling pathways confer resistance in the cells chronically exposed to entospletinib in comparison...
**Figure 3.** Validation of ORF screen results. Overexpression of RAS confers resistance to SYK inhibition. Immunoblot confirming overexpression of the indicated ORF hits in MV4-11 cells (A) and MOLM14 cells (B) by antibodies directed against the ORF or downstream effectors. α-tubulin and vinculin were used as loading controls. C and D, Long-term viability assays in MOLM14 (left) and MV4-11 (right) cells overexpressing the indicated ORFs and treated with vehicle or 3 μmol/L entospletinib. GFP is included as a negative control. Data are presented as mean values of triplicate replicates ± SD. E, Immunoblot confirming overexpression of NRASG12D, as well as activation of the downstream effector phospho-ERK1/2, in retrovirally transduced murine MLL-AF9 (MAF9) rearranged leukemia cells. Vinculin was used as a loading control. F, Viability analysis with increasing concentrations of entospletinib for 72 hours in MAF9 leukemia cells. Data are plotted as the percentage of luminescence (measured by CellTiter-Glo) relative to DMSO controls. G, Viability analysis with increasing concentrations of entospletinib in a panel of 12 different AML cell lines after 72 hours of treatment. H, Average difference in AUC drug response between mutant and wild-type cases of 239 primary patient AML samples was determined using a Student two-sided t-test from a linear model fit (x-axis). P values were corrected using the Benjamin–Hochberg method over all genes, −log10(FDR) values are plotted (y-axis). Sensitive samples are red; resistant samples are blue. Size of circles correlates with patient sample size. Count, number of patients. Data were generated using the online data tool www.vizome.org (11).
**Figure 4.** Characterization of MV4-11 cells with acquired entospletinib resistance. A and B, Viability analysis after 72 hours of entospletinib (A) or PRT062067 (B) treatment in naïve and resistant MV4-11 cells. Data are plotted as the percentage of luminescence (measured by CellTiter-Glo) relative to DMSO controls. C, Immunoblot demonstrating upregulated phospho sites of downstream effectors of SYK in the resistant state. Specific antibodies to indicated phospho sites were used. GAPDH was used as a loading control. D, Heat map demonstrating upregulation of expression of indicated genes in replicate resistant cells (right) in comparison with replicate naïve cells (left). Significance was assessed on the basis of log2 fold-change expression ≥ 0.5 and P ≤ 0.05 for the Mann–Whitney test. E, Confirmation of NRAS and KRAS mRNA fold-change upregulation in resistant versus naïve MV4-11 cells by qPCR (***, P ≤ 0.001, Mann–Whitney test). F, GSEA demonstrating enrichment of signatures upregulated in resistant cells in comparison with naïve cells. Significance was assessed on the basis of NES ≥ 1.5, P ≤ 0.05, and FDR ≤ 0.05.
with naive cells, we evaluated the main downstream effectors of pathways that scored in the ORF screen: JAK–STAT, AKT–mTOR, and RAS–MAPK–ERK. Strikingly, we detected an increase in phospho-SYK signaling (Fig. 4C), indicating that in the resistant cells, activation of parallel signaling pathways results in a feedback activation of SYK phosphorylation sites. Moreover, we detected increased phosphorylation in each of the three pathways scoring in the screen (ERK1/2, STAT5, STAT3, and SHP2), suggesting that cells resistant to entospletinib have adapted to drug exposure by upregulation of the primary target SYK, as well as other signaling pathways bypassing SYK (Fig. 4C), such as JAK–STAT (phospho-STAT3) and RAS–MAPK–ERK signaling (phospho-SHP2, phospho-ERK1/2, and total RAS). We validated these findings in another population of MV4-11 cells independently grown to resistance with chronic entospletinib treatment (Supplementary Fig. S4A and S4B).

As a next step, we investigated whether these changes in signaling were associated with a change in gene expression using RNA-seq analysis of the MV4-11 resistant cells. We observed a significant upregulation of \( \text{NRAS} \) and \( \text{KRAS} \) (\( P < 0.05 \)), as well as the guanine nucleotide exchange factors \( \text{SOS2} \) and \( \text{RASGRF1} \), which couple signals from protein tyrosine kinases to RAS, facilitating the GDP–GTP exchange (refs. 38, 39, Fig. 4D). Moreover, we detected upregulated expression of genes encoding downstream effectors [\( \text{BRAF}, \text{MAP3K1} \) (encoding MEKK1) and \( \text{MAPK3} \); Fig. 4D]. We validated the upregulation of \( \text{NRAS} \) and \( \text{KRAS} \) by RT-PCR (Fig. 4E). In line with these observations, the gene sets for KRAS and mTOR signaling were enriched by GSEA in the SYK inhibitor–resistant cells compared with the drug-sensitive cells (Fig. 4F).

We next explored whether there was clonal heterogeneity in this resistant population of cells and selected four single-cell clones (SCC) from the bulk resistant cells by serial dilution. Only two out of four clones showed a similar increase in phosphorylation signals (Supplementary Fig. S4C) to that observed in the bulk population of resistant cells (upregulation of phospho-SYK, phospho-ERK1/2, phospho-SHP2, and RAS). Importantly, each of these single-cell selected populations were resistant to entospletinib (Supplementary Fig. S4D), consistent with clonal heterogeneity in these cells. These data suggest that in our acquired SYK inhibitor resistance model, upregulation of the RAS–MAPK–ERK pathway is a dominant resistance mechanism, but there are also other resistance mechanisms at play.

To further investigate mechanisms underlying resistance in these MV4-11 cells, we performed whole-exome sequencing (WES) of the drug-sensitive versus drug-resistant cells. We did not observe new mutations in \( \text{SYK}, \text{RAS} \), or other receptor tyrosine kinases (RTK; Supplementary Table S7). And, although we observed a mutation in the E3 ligase C-CBL, this occurred in only one of the two populations of cells and with a variant allele frequency (VAF) of 24%, explaining at best resistance in a minor population of cells. Similarly, we did not identify any copy gain in RAS–MAPK–ERK pathway members to explain the increase in transcripts such as \( \text{KRAS}, \text{NRAS} \), or \( \text{BRAF} \), nor the activation of the MAPK pathway (Supplementary Table S8).

Taken together, these data support a model whereby subclonal, largely nongenetic events (e.g., increase in RAS expression) contribute to the resistance observed in the bulk populations of MV4-11 resistant cells.

**Activation of the RAS–MAPK–ERK Pathway Is Associated with Resistance in Patients with AML**

To explore whether our findings are also of importance in patients, we retrospectively analyzed a cohort of patients with AML treated with entospletinib. Twenty-eight patients were treated with entospletinib as part of a phase Ib/II study (200 or 400 mg twice a day) for 14 days prior to receiving concurrent conventional 7 + 3 chemotherapy. Three patients whose AML harbored \( \text{PTPN11} \) mutations as part of their mutational profile (Fig. 5A) were enrolled. Each of these patients either failed to attain a remission or relapsed within 1 month of complete morphologic remission (CR) with mediullary and/or extramedullary disease. To functionally validate the mutations observed in primary patient AML cells, we used site-directed mutagenesis to generate activating \( \text{PTPN11} \) mutations (E76G and T73I) that were observed in two of these patients. First, we confirmed overexpression in MV4-11 cells by immunoblotting (Fig. 5B). Next, we cultured the cells chronically for three weeks with entospletinib in vitro. The \( \text{PTPN11} \) mutants did not confer a growth advantage to the control cells in DMSO (Supplementary Fig. S5A). However, both \( \text{PTPN11} \) mutations rendered resistance to entospletinib in comparison with the control cells overexpressing GFP or wild-type \( \text{PTPN11} \) (Fig. 5C). Importantly, we observed a failure to repress phospho-ERK1/2 with entospletinib treatment in the \( \text{PTPN11} \) mutant context in line with SYK inhibitor resistance (Fig. 5D). Moreover, for the one patient whose relapse skin biopsy had a sample sufficient to obtain interpretable sequencing data, the original \( \text{PTPN11} \) and \( \text{NPM1} \) mutations were identified in addition to novel, previously uncharacterized \( \text{PTPN11} \) and MAPK1 mutations (Supplementary Fig. S5B). However, neither of these new mutations has been reported previously, and their functional relevance remains unknown. Therefore, both mutations will require future validation and functional characterization.

**Inhibitors of the RAS–MAPK–ERK Pathway Synergize with SYK Inhibition to Overcome RAS-Mediated Resistance**

Because RAS-mutant AML cells are sensitive to MEK inhibition (40, 41), we next tested whether MEK inhibition using PD0325901 (42) would recaputure response to SYK inhibition in RAS–MAPK–ERK–activated AML cells resistant to entospletinib. The combination of entospletinib and PD0325901 demonstrated strong synergy based on the Chou–Talalay combination index (CI) model (43) in RAS-mutant cell lines. We could detect strong synergy in \( \text{NRAS}^{G12D} \) mutant THP-1 (Fig. 6A) and in the primary murine \( \text{MLL}-\text{AP2} \) leukemia bone marrow cells retrovirally transduced with a plasmid containing mutated \( \text{NRAS}^{G12D} \) (Fig. 6A). We could also detect strong synergy if RAS is activated by rewiring of the signaling pathways as in the MV4-11 cells with acquired resistance to SYK inhibitors (Fig. 6A). In the MV4-11 cells with acquired resistance, the combination of entospletinib and PD0325901 led to cell death (Supplementary Fig. S6A). Culturing of cells with PD0325901 in combination with entospletinib was also able to resensitize other RAS–MAPK–ERK-activated cell lines to
SYK inhibition, resulting in increased cell death (Supplementary Fig. S6A). Next, we confirmed that synergy persists if RAS is activated by mutation of an upstream effector (U937 cell line expressing PTPN11G60R; Fig. 6A). Isobolograms revealed synergy across a wide range of concentrations. Strikingly, we also saw additivity/synergy of the combination of entospletinib and PD0325901 in the genetically engineered cell lines (Fig. 6A; Supplementary Fig. S6B) harboring PTPN11 wild-type or PTPN11 with mutations (E76G and T73I) and in the RAS wild-type AML cell lines MV4-11 and MOLM14 (Supplementary Fig. S6B).

We extended this evaluation to a primary AML sample (Fig. 6A) possessing NRASG12D, KRASG12D, and PTPN11G60R mutations obtained from a patient with myeloproliferative neoplasm where we similarly saw additivity/synergy depending on the concentrations tested. We confirmed strong synergy in two additional samples obtained from patients with RAS wild-type AML (Fig. 6A). We next confirmed synergistic activity in three previously tested PDX models of AML in vitro (Supplementary Fig. S6B).

To confirm our results in an in vivo context, we evaluated the tolerability of the combination of entospletinib and PD0325901. We treated healthy C57BL/6 mice with entospletinib, PD0325901, the combination, or vehicle for 28 days, and assessed the impact on different organs. We did not observe any toxic effect on the hematopoietic system (white blood count, neutrophils, hemoglobin, platelets; Fig. 7A), nor on liver or kidney function (total bilirubin, ALT, BUN, creatinine; Supplementary Fig. S7A). Moreover, we did not observe any toxic effects on bone marrow (Supplementary Fig. S7B), liver or kidney tissue (Supplementary Fig. S7C) in hematoxylin and eosin (H&E) staining, nor did the mice show any significant weight loss during treatment (Supplementary Fig. S7D).

Next, to evaluate a PDX model in vivo (PDX #2, MLL-AF4 rearrangement), mice were treated with either vehicle,
entospletinib, PD0325901, or the combination of entospletinib and PD0325901 (Fig. 7B). Mice were sacrificed after 14 days of treatment, and the leukemia burden evaluated by detection of hCD45-positive cells by flow cytometry. We observed a significant reduction in leukemia burden in the bone marrow of the mice when comparing the vehicle-treated mice with each other treatment group (Fig. 7B), and also when comparing the entospletinib-treated mice with the mice that received combination treatment ($P \leq 0.05$; Fig. 7B). As had been observed with the PDX #1 and PDX #3, entospletinib alone prolonged survival of the mice ($P \leq 0.0001$). However, there was a greater survival advantage in the combination-treated group in comparison with all other treatment groups (Fig. 7C).

Finally, we investigated whether the combination treatment is also efficacious in a RAS-mutant context in vivo. C57BL/6 mice, injected with very aggressive murine MLL-AF9/NRAS<sup>G12D</sup> cells, were treated for 14 days with entospletinib, PD0325901, combination, or vehicle. The combination therapy had a superior effect over single-drug treatment with regard to reduction of GFP-positive leukemic cells in the bone marrow, peripheral blood, and spleen (Fig. 7D). Strong differences of spleen sizes between the mice in the different treatment groups were also observed (Fig. 7E).
Figure 7. Combination of a MEK inhibitor and a SYK inhibitor is active in vivo AML models and shows no toxicity in healthy mice. A, Complete blood count was evaluated after 14 days of treatment in healthy C57BL/6 mice. WBC, whole blood count; Ne, neutrophils; Hb, hemoglobin; Plt, platelets. B, Leukemia burden was assessed with flow cytometry by measuring human CD45-positive cells/total live cells by in the bone marrow (BM) of NSG mice injected with PDX #2 and treated with vehicle, entospletinib, PD0325901, or the combination of entospletinib and PD0325901 for 14 days. Data are plotted as mean values ±SEM (n = 6–7 mice per arm; ****, P ≤ 0.0001; *, P = 0.05). Significance was determined by two-way ANOVA. C, Survival study in NSG mice, injected with PDX #2, randomly assigned to entospletinib, PD0325901, combination, or vehicle treatment, n = 8 mice per arm (****, P ≤ 0.0001; *, P ≤ 0.05, Mantel–Cox test). D, Leukemia burden was assessed by measuring GFP-positive cells/total live cells by flow cytometry in the BM (left), peripheral blood (PB; middle), or spleen (SPL; right) of C57BL/6 mice injected with primary murine MLL-AF9 leukemia bone marrow cells retrovirally transduced with a plasmid containing mutated NRAS<sub>G12D</sub> and treated with vehicle, entospletinib, PD0325901, or the combination of entospletinib and PD0325901 for 14 days. E, Spleen weights from the four treatment groups. Data are plotted as mean values ±SEM (n = 4–6 mice per arm; ****, P ≤ 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05). Significance was determined by two-way ANOVA.
These results suggest that the combination of a MEK inhibitor and a SYK inhibitor is broadly effective in \textit{in vitro} and \textit{in vivo} AML models and results in an improvement of survival, identifying this combination as a potential strategy that is well tolerated \textit{in vitro} and might be able to block emergent resistance driven by activation of the RAS–MAPK–ERK signaling pathway. Furthermore, our findings suggest that targeting downstream effectors of RAS signaling in the MAPK signaling family is able to overcome resistance to SYK inhibition in AML.

**DISCUSSION**

The most common mechanism of acquired resistance to targeted therapies is the development of secondary mutations in the drug target. SYK, however, is rarely mutated, which makes it different from other targets, such as FLT3. Because SYK inhibitors are now in clinical trials for the treatment of patients with AML where early promising data have been observed when combined with traditional therapies, we set out to identify the mechanisms of resistance by performing a proteogenomic characterization of SYK inhibitor resistance.

We identified the activation of RAS signaling as a major mechanism of resistance to SYK inhibition in AML, through mutations in \textit{RAS} and \textit{PTPN11} in preclinical models of AML, as well as by rewired signaling at least in part through altered gene expression in AML cell lines. Intriguingly, \textit{PTPN11} mutations were also observed in patients with AML with early relapse treated with a SYK inhibitor. \textit{RAS} genes are proto-oncogenes that are mutated in approximately 15% of adult AML (31) and in more than 30% of pediatric AML (33). In addition to mutations in \textit{RAS} itself, \textit{RAS} signaling can be deregulated by mutations in genes that activate the signaling pathway, such as \textit{FLT3} and \textit{c-KIT}, in another 25% to 40% of patients with AML (44). Studies of patients with juvenile myelomonocytic leukemia (JMML) also underscore the importance of hyperactivated \textit{RAS} signaling in myeloid malignancies. It is known that somatic mutations in \textit{PTPN11}, which potentiate \textit{RAS} signaling, occur in approximately 30% of patients with JMML (37, 45) and in 7% of patients with AML (36), and in 10% to 15% of JMML and 1% to 3% of AML, respectively.

Although the prognostic significance of \textit{RAS} mutations remains debated in the context of standard cytotoxic chemotherapy (32, 46, 47), when selective pressure is applied to AML cells, \textit{RAS} family mutations may bear therapeutic relevance. For example, in patients with AML treated with the selective mutant IDH2 inhibitor enasidenib, \textit{NRAS} or \textit{PTPN11} mutations are associated with decreased response rates (48). In addition, in a large study of primary patient samples treated \textit{ex vivo} with more than 100 small-molecule inhibitors, mutations in \textit{NRAS} or \textit{KRAS} were frequently associated with attenuated drug response (11). By putting selective pressure on AML cells with entospletinib treatment, the presence of \textit{RAS} pathway mutations has also emerged as a biomarker of resistance in our study.

Despite the promising single-agent activity of very potent targeted therapies, such as the FLT3 inhibitor quizartinib, most patients relapsed within three months (49, 50). The main categories of resistance to targeted therapies are secondary mutations of the target, activation of other upstream stimulators or downstream effectors of the pathway, or the activation of parallel pathways (51). In the case of secondary mutations in the target itself, the development of new inhibitors may be called for. In the case of activation of parallel signaling pathways or the activation of other upstream activators or downstream effectors of the pathway, the combination of targeted drugs is critical to shutting down the escape mechanisms (52). The discovery of drug combinations that are able to prevent the emergence of resistance is an unmet clinical need in AML and targeted cancer therapy more broadly. Genome-scale functional genomic screens provide a powerful, unbiased approach to identify drug resistance mechanisms (53) preemptively even before the clinical trial is initiated. Such an approach has been used successfully in the past to predict emerging mechanisms of resistance to targeted therapies (23, 54). For example, a gain-of-function screen identified \textit{COT} mutations as drivers of resistance to RAF inhibitor treatment in \textit{BRAF\textsubscript{V600E}}-mutant melanoma cell lines, as well as in patients who received treatment with MEK or RAF inhibitors (23). Here, we provide further support that these screening efforts can predict resistance mechanisms observed in the clinic.

As in a recent publication describing a complex pattern of leukemia heterogeneity and clonal evolution under selection pressure with gilteritinib, we too see clonal heterogeneity in AML cells grown under chronic entospletinib treatment (55). In two independent MV4-11 cell populations, we identified hyperactivation of the SYK–MAPK pathway. At the level of single-cell clones, however, we observed variability in the degree of SYK–MAPK pathway activation despite all of the clones retaining entospletinib resistance. In line with this observation, although we did not see new RTK/MAPK mutations or copy-number alterations in the resistant cells, in one of the two populations of MV4-11 drug-resistant cells, we detected a subclonal mutation in the E3 ligase CBL, which would be predicted to result in entospletinib resistance. Similarly, CBL mutations were reported in the context of gilteritinib resistance (55). Additional studies will be needed to determine the precise mechanism by which the drug-resistant cells gain an increased expression of MAPK-related genes. In our preliminary studies, we do not see alterations in H3K27ac levels nor in chromatin accessibility to explain the gene-expression changes (data not shown). Future studies will evaluate whether there are changes in repressive marks, chromatin looping, or the pattern of transcription factors binding at the promoter/enhancer regions for these genes.

Because of the structural and biochemical properties of RAS, it is not yet possible to target RAS directly (30), but it is known that \textit{RAS}-mutant cell lines are sensitive to MEK inhibition (40). Because we identified activation of the RAS signaling pathway as the major mechanism of resistance to SYK inhibition in our study, we tested the hypothesis that the combination of a MEK and a SYK inhibitor would show synergy \textit{in vitro}. In fact, we demonstrated synergistic activity in combining PD0325901 with entospletinib in a panel of \textit{RAS}-mutated cell lines, primary patient samples, as well as a syngeneic \textit{MLL-AF9} primary leukemia cell model. Importantly, this drug combination...
maintained synergistic activity even in RAS wild-type cells and resulted in a greater reduction in leukemia burden compared with single-agent treatments in a PDX AML model.

The selective, oral, allosteric MEK1/2 inhibitor trametinib was recently FDA-approved as part of the standard of care for patients with metastatic \textit{BRAF}-mutant melanoma (56). To date, data from two clinical trials testing MEK inhibitors in patients with AML have been published. In a phase II study, selumetinib showed modest single-agent antileukemic activity in patients with advanced leukemia (57). Another study tested the single-agent activity of trametinib in patients with relapsed or refractory AML. In this study, patients with AML with \textit{NRR} or \textit{KRAS} mutations were enrolled in Cohort 1 and patients without \textit{RAS} mutations into Cohort 2. Patients in Cohort 1 had an overall response rate of 20% compared with 3% in Cohort 2 (58). The most common drug-related toxicities were grade 1 to 2 diarrhea, fatigue, vomiting, and skin rash (57, 58). Both studies concluded that although some single-agent activity was observed with a MEK inhibitor in AML, combination with another therapy in patients with AML should be considered. Our results suggest that entospletinib, which is very well tolerated in patients, is a drug to consider in combination with MEK inhibitors in AML, both in \textit{RAS}-mutant and RAS wild-type disease, and should be further investigated in additional preclinical \textit{in vivo} models.

SHP2 activation has previously been identified as one major node in acquired and intrinsic resistance in cancer cells driven by RTKs (59). We identified SHP2 as one of the proteins whose phosphorylation was most significantly downregulated in our pYome analysis with SYK inhibitor treatment. After short-term treatment of AML cells with entospletinib, we detected decreased phosphorylation of SHP2 at pY546, which is located in close proximity to the major phosphorylation site (Y542) of SHP2. Indeed, SHP2 activation is required for the full activation of the RAS–MAPK–ERK signaling pathway (60). Furthermore, SHP2 was identified as a direct activator of RAS and a potential target for cancers driven by hyperactive or oncogenic RAS (61). SHP2 recruitment to duplicated Y599 on FLT3-ITD cooperates with SYK to enhance STAT5 phosphorylation and promote ERK phosphorylation (12). Our patient data suggest an association of \textit{PTPN11} mutations with early relapse, and we demonstrate that these mutations render resistance to SYK inhibitors in AML cell lines treated \textit{in vitro}. Although these data are provocative, more research is needed to definitively demonstrate that the \textit{PTPN11} mutations cause resistance in patients with AML treated with SYK inhibitors. For example, serial samples of high quality to enable confident assessment of VAF of \textit{PTPN11} mutations in patients treated with SYK inhibitors and functional validation of the novel \textit{PTPN11} and \textit{MAPK1} mutations identified in the current study are needed.

Recently, the first orally bioavailable highly potent allosteric \textit{PTPN11} inhibitor, SHP099, was discovered (62, 63). SHP099 treatment led to a reduction in leukemia burden in an orthotopic human primary leukemia–derived \textit{FLT3-ITD} AML model (62), suggesting that Shp099 could be a promising candidate for future drug combination studies in AML. Two recent reports identified a combination of a \textit{PTPN11} inhibitor with a MEK inhibitor as a targeted therapy approach not only in RAS wild-type cancers, but also in \textit{KRAS}-mutant and \textit{KRAS}-amplified cancers (52, 64). Taken together, these data suggest that the combination of Shp099 and entospletinib is a drug combination worth investigating in future experiments.

In conclusion, we report that RAS pathway activation, through \textit{RAS}, \textit{PTPN11}, and potentially CBL mutations, or upregulation of expression of these target genes, renders resistance to SYK inhibitors in AML in preclinical models and was observed in patients treated with the SYK inhibitor entospletinib with early relapse. Furthermore, we provide preclinical data suggesting that combining SYK with MEK inhibitors has the potential to prevent the emergence of resistance to SYK inhibition and even shows up-front efficacy. Our approach exemplifies how a large-scale genomic screen may contribute to delineating clinical exclusion criteria for a targeted inhibitor, in this case \textit{RAS} or \textit{PTPN11} mutations, and to determine effective drug combinations that may prevent the development of resistance.

**METHODS**

**Cell Lines**

U937, HL-60, MOLM13, UCSD-AML1, NB4, NOMO-1, and 293T cells were purchased from ATCC. CMK-86 cells were purchased from JCRB Cell Bank. EOL-1 cells were purchased from DSMZ. MV4-11, MOLM14, and THP-1 cells were provided by Scott Armstrong. MOLM13 and MOLM14 cells were originally derived from the same patient. All cell lines were maintained in RPMI-1640 (Cellgro) supplemented with 1% penicillin/streptomycin (Cellgro) and 10% FBS (Sigma-Aldrich) at 37°C with 5% CO₂. The 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. All cell lines were tested negative for \textit{Mycoplasma} and were authenticated using short tandem repeat profiling.

**Western Blotting**

Proteins were extracted using Lysis Buffer (Cell Signaling Technology) supplemented with Complete, Ethylenediaminetetraacetic Acid (EDTA)-Free Protease Inhibitor Cocktail (Roche Diagnostics) and Phosphatase Inhibitor Cocktail (Roche Diagnostics). Protein samples were separated by SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes, which were blocked in 5% BSA and incubated with primary antibodies against RAS protein (Cell Signaling Technology catalog no. 14429), RAS (Cell Signaling Technology catalog no. 3965), NRAS (Santa Cruz Biotechnology catalog no. sc-31), KRAS (Santa Cruz Biotechnology catalog no. sc-30), HRAS (Santa Cruz Biotechnology catalog no. sc-35), phospho-STAT5 Y694 (Cell Signaling Technology catalog no. 9351), STAT5 (Cell Signaling Technology catalog no. 9363), phospho-STAT3 (Cell Signaling Technology catalog no. 9145), STAT3 (Cell Signaling Technology catalog no. 9139), phospho-p44/42 MAPK T202/Y204 (Cell Signaling Technology catalog no. 9101), p44/42 MAPK (Cell Signaling Technology catalog no. 4696), phospho-SHP2 Y542 (Cell Signaling Technology 3751), SHP2 (Cell Signaling Technology catalog no. 3397), phospho-SYK Y525/Y526 (Cell Signaling Technology catalog no. 2711), SYK (Santa Cruz Biotechnology catalog no. sc-1240), vinculin (Abcam catalog no. 18058), GAPDH (Santa Cruz Biotechnology catalog no. sc-47724), and α-tubulin (Calbiochem, catalog no. CP06). Phospho-protein signals were normalized to total protein content, which was normalized to δ-actin (Cell Signaling Technology catalog no. 4696), phospho-SHP2 Y542 (Cell Signaling Technology 3751), phospho-SYK Y525/Y526 (Cell Signaling Technology catalog no. 2711), SYK (Santa Cruz Biotechnology catalog no. sc-1240), vinculin (Abcam catalog no. 18058), GAPDH (Santa Cruz Biotechnology catalog no. sc-47724), and α-tubulin (Calbiochem, catalog no. CP06). Phospho-protein signals were normalized to total protein content, which was normalized to δ-actin (Cell Signaling Technology catalog no. 4696), phospho-SHP2 Y542 (Cell Signaling Technology 3751), phospho-SYK Y525/Y526 (Cell Signaling Technology catalog no. 2711), SYK (Santa Cruz Biotechnology catalog no. sc-1240), vinculin (Abcam catalog no. 18058), GAPDH (Santa Cruz Biotechnology catalog no. sc-47724), and α-tubulin (Calbiochem, catalog no. CP06).
secondary antibodies. Signal was detected by enhanced chemiluminescence (Thermo Fisher Scientific).

**SILAC**

For SILAC, cells were cultured in SILAC-RPMI devoid of arginine and lysine (Thermo Fisher Scientific) supplemented with 10% heat-inactivated dialyzed FCS (Sigma-Aldrich), 100 U/ml penicillin/100 mg/ml streptomycin (Life Technologies), and amino acids with heavy isotopes (13C6, 15N4) or regular (light) amino acids (12C6, 14N4) in a 96-well plate, at 1,500 rpm for 2 hours at room temperature. Lentiviral was generated using the packaging plasmids delta8.9 and VSVG, following the X-tremeGENE HP DNA Transfection Reagent protocol (Roche). AML cells were infected with 2 μl of virus and 8 μg/ml polybrene. Retrovirus was generated using the packaging plasmids, y6eco and RV. Primary murine bone marrow cells were infected by spin-infection in a centrifuge with 50 μl of virus and 8 μg/ml polybrene in a 96-well plate at 1,500 rpm for 2 hours at room temperature. Cells were selected with puromycin-containing media 48 hours after infection.

**Generation of MLL-AF9 Primary Mouse Leukemia Cells**

Primary MLL-AF9 leukemia cells were provided by S.H. Chu. LIN KIT+SCA1+ hematopoietic stem cells were sorted from mice 6 to 8 weeks of age by harvesting of bone marrow cells from the femurs, tibia, hips, and spines as described previously (65) and retrovirally transduced with a MSCV-IRES-MLL-AF9-GFP construct. Cells were maintained in 15% Iscove’s Modified Dulbecco Medium supplemented with penicillin/streptomycin, murine IL6, murine SCF, and murine IL3 (as above) for 2 days before FACS for GFP-positive cells.

**Generation of Entospletinib-Resistant Cells**

To generate cells that were resistant to SYK inhibition, MV4-11 cells were treated for 5 months with gradually increasing concentrations of entospletinib (500 nmol/L to 5 μmol/L). Cells were considered entospletinib-resistant when they were able to remain 90% to 100% viable in the presence of this 10-fold higher than IC50 concentration of entospletinib.

**Site-Directed Mutagenesis**

The different point mutations in the PTPN11 sequence were generated according to the manufacturer’s instructions using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). All sequences were confirmed by Sanger sequencing. The following primers were used: PTPN11 T73I, Forward: AGGGGAGAAATTTGC CATTTCGTGCTAGTGTGTCCA, Reverse: TGGAGCAACTCAGC CAAAATGGGCAATTTCCTCCCT. PTPN11 E76G, Forward: ATTT GCCACTTTTGGCTGGTGGTCCAGTATTACAT, Reverse: ATG TAATACTGGAAC-ACCCAGCACAATGGGCAAT.

**In Vivo Transplantation**

All in vivo studies were conducted under the auspices of protocols approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Six- to 8-week-old female NSG mice or C57BL/6 mice (The Jackson Laboratory) were injected with 1 × 106 cells. Bone marrow was harvested from femur, tibia, and spine, and red blood cells were lysed (Sigma) prior to staining with human CD45 (Invitrogen catalog no. MHCID4528) and murine CD45 (BioLegend catalog no. 103113) antibodies and analysis by flow cytometry.

**In Vivo Therapeutic Studies**

Animals were randomly assigned to experimental treatment groups without binding at any stage of the study. Treatment was started after we assessed engraftment by flow cytometry (human CD45+ leukemic cells or GFP-positive cells in the peripheral blood ≥1%). For efficacy studies, leukemia burden was assessed using flow cytometry analysis of the percent of human CD45+ leukemic cells in the bone marrow on day 14 after treatment start. PD0325901 was administered once daily by oral gavage. We started dosing at 5 mg/kg body weight but needed to reduce the dose to 2.5 mg/kg body weight after three days of treatment due to weight loss of the mice in the PD0325901 and combo treatment arms. We continued with a 5 days on, 2 days off schedule for the remaining 10 days. For the entospletinib survival study, mice were weighed daily and were sacrificed when they reached a 20% weight loss threshold and/or showed lethargy/physical signs of illness. All animals assigned to treatment groups were included in the survival analysis.

**In Vivo Toxicity Study**

Healthy C57BL/6 mice were treated for 28 days with vehicle, entospletinib, PD0325901, or a combination of both drugs. We assessed complete blood counts at 14 days, and BUN, creatinine, total bilirubin, and ALT at 28 days. Moreover, weight of the mice was monitored over time. Bones, kidney, and liver were harvested at the end of the study and H&E staining of representative animals from each group was performed.

**Primary Patient Samples**

The three patients with AML reported in this article were treated with entospletinib in a clinical trial at Ohio State University (Columbus, OH). Patient samples used for synergy studies were obtained from patients treated at the Dana-Farber Cancer Institute (Boston, MA) after obtaining informed consent under Dana-Farber Cancer Institute Internal Review Board–approved protocols. AML blasts from patients were maintained in Serum-Free Expansion Medium (StemCell Technologies) with SCF, FLT3L, IL3, IL6, and G-CSF (PeproTech). All patients provided written informed consent, and the studies were conducted in accordance with the Declaration of Helsinki and after Human Research Ethics Committee approval.

**Chemicals**

All compounds for in vitro experiments were obtained from Selleck. PD0325901 for in vivo experiments was obtained from Selleck. Entospletinib chow for in vivo experiments was provided by Gilead.

**Validation of ORF Hits**

Lentivirus-infected cells expressing candidate ORF hits from the primary screen were seeded in T25 flasks in technical duplicate and were treated with DMSO or 3 μmol/L entospletinib. Cumulative population doublings were calculated by manually counting cells every 3 to 4 days for a total of 21 days.

**ORFeome Library Titration**

Accurate virus volumes to use in large scale were determined in each cell line to achieve 30% to 40% infection efficiency, corresponding to a multiplicity of infection (MOI) of approximately 0.5 to 1. Spin infections were performed in 12-well plate format with 3 × 104 and 1.5 × 105 cells per well for MV4-11 and MOLM14 cells, respectively, with different virus volumes (0, 100, 200, 300, 400, 500 μL) with a final concentration of 8 μg/ml polybrene. Cells were spin-infected for 2 hours at 2,000 rpm at 30°C. Twenty-four hours later, cells were harvested and 2 × 106 MV4-11 cells or 3.5 × 105 MOLM14 cells from each infected were seeded into duplicate wells in 6-well plates, each with complete medium and one treated with puromycin. Seventy-two to 96 hours after selection, cells were counted to determine the
amount of virus that yielded approximately 30% to 40% infection efficiency, and this amount was used for screening.

**Genome-Scale ORF Screens**

The ORFeome barcoded library contains 17,255 barcoded ORFs overexpressing 10,135 distinct human genes with at least 99% nucleotide and protein match. Screening-scale infections of the ORFeome library were performed with a number of cells to achieve a representation of at least 1,000 cells per ORF (~2 × 10^5 surviving cells containing 17,255 ORFs). Infections were performed with the predetermined virus volume in the 12-well format, as the viral titration described above, and pooled 24 hours postinfection. Approximately 24 hours after infection, all wells within a replicate were pooled and 48 hours after infection, cells were selected with puromycin. After selection was completed, 3 × 10^7 cells were divided into drug-treated (3 μmol/L entospletinib for MV4-11 and MOLM14) and vehicle-treated arms. Cells were passaged in drug or fresh media containing drug every 3 to 4 days, and throughout the screen we maintained an average representation of 1,000 cells per ORF construct. Cells were harvested 21 days after initiation of treatment. For both ORF screens, gDNA was isolated using Maxi (2 × 10^7 to 1 × 10^8 cells) or Midi (5 × 10^6 to 3 × 10^7 cells) Kits according to the manufacturer’s protocol (Qiagen). PCR and sequencing were performed as described previously (28). We performed two replicates for the ETP and two replicates for the late time point for each cell line.

**RNA-seq**

RNA was extracted from cells with the RNeasy Kit and on-column DNA digestion (Qiagen). For RNA-seq of MV4-11 cells, poly(A) mRNA was isolated and libraries were prepared using the TruSeq Stranded mRNA Kit (Illumina) according to the manufacturer’s protocol. All samples were sequenced on a NextSeq 500 instrument with single-end 75 bp reads to a depth of 30 to 50 M reads/sample.

**GSEA**

GSEA v3.0 software was used to identify functional associations of the genome-wide molecular profiles. Significance cutoffs were assessed on the basis of the GSEA standard recommendations: absolute NES ≥ 1, P ≤ 0.05, Benjamini–Hochberg FDR ≤ 0.25.

**Cell Viability and Synergy Studies**

Cells were resuspended at 15,000 cells/mL and seeded at 40 μL/well into 384-well plates. Cells were then treated with a single agent or combination of compounds and analyzed for cell viability on days 0 and 3 post-treatment using the CellTiter-Glo Luminescent Assay Kit (Promega) according to the manufacturer’s protocol. Luminescence was read on a Fluostar Omega Reader (BMG Labtech).

**Quantification and Statistical Analyses**

GSEA v2.1.0, GraphPad PRISM 7, R 3.2.3, and Python 2.7.2 software packages were used to perform the statistical analyses. Statistical tests used are specified in the figure legends. Error bars, SD, unless otherwise stated. The threshold for statistical significance is P ≤ 0.05, unless otherwise specified.

**Data Availability**

RNA-seq data was deposited at the Gene Expression Omnibus (GEO) under accession number GSE129698. WES data was deposited at NCBI-SRA under accession number PRJNA532457 (WES Data1) and PRJNA565774 (WES Data2). For detailed information on RNA-seq data processing, synergy analysis, enrichment of phosphopeptides, mass spectrometry, serial dilution assay, WES analysis, somatic variant calling/analysis, and data processing, see Supplementary Methods.

**Disclosure of Potential Conflicts of Interest**

A.V. Rao is executive director, clinical research, at Kite-Gilead and has ownership interest (including patents) in the same. J.S. Blachly is an advisor at Abbvie, AstraZeneca, Innate Pharma, and Kite Pharma and has received other remuneration from Oxford Nanopore Technologies. S.A. Armstrong is a consultant at Epizyme, Imago, C4 Therapeutics, Cyteir Therapeutics, and Allergan, is a SAB member at OxStem Oncology, Accent Therapeutics, and Mana Therapeutics, and reports receiving commercial research grants from Novartis, Janssen, and AstraZeneca. K. Stegmaier is a consultant at Rigel Pharmaceuticals, reports receiving a commercial research grant from Novartis, and has an unpaid consultant/advisory board relationship at Gilead. No potential conflicts of interest were disclosed by the other authors.

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**Development of methodology:** A. Cremer, J.M. Ellegast, S.H. Chu, A. Goodale, F. Piccioni, J.C. Byrd, K. Stegmaier

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A. Cremer, J.M. Ellegast, L. Ross, S.H. Chu, Y. Pikman, A. Robichaud, A. Goodale, B. Haupl, S. Mohr, A.V. Rao, A.R. Walker, J.S. Blachly, F. Piccioni, J.C. Byrd, K. Stegmaier

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Cremer, J.M. Ellegast, G. Alexe, E.S. Frank, Y. Pikman, B. Haupl, J.S. Blachly, F. Piccioni, J.C. Byrd, K. Stegmaier

**Writing, review, and/or revision of the manuscript:** A. Cremer, J.M. Ellegast, G. Alexe, Y. Pikman, A.V. Rao, A.R. Walker, J.S. Blachly, S.A. Armstrong, J.C. Byrd, T. Oellerich, K. Stegmaier

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.M. Ellegast, R. Haupl

**Study supervision:** A. Cremer, T. Oellerich, K. Stegmaier

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**REFERENCES**


Resistance Mechanisms to SYK Inhibition in AML


# Resistance Mechanisms to SYK Inhibition in Acute Myeloid Leukemia

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