Targeting BCL-2 and ABL/LYN in Philadelphia chromosome–positive acute lymphoblastic leukemia

Jessica T. Leonard,1 Joelle S. J. Rowley,2 Christopher A. Eide,2,3 Elie Traer,1,2 Brandon Hayes-Lattin,1,2 Marc Loriaux,2,4 Stephen E. Spurgeon,1,2 Brian J. Druker,2,3 Jeffrey W. Tyner,2,5* Bill H. Chang2,6*

INTRODUCTION

Philadelphia chromosome–positive acute lymphoblastic leukemia (Ph+ALL) is an aggressive subtype of ALL with a dismal prognosis. Before 2001, the diagnosis of Ph+ ALL in adults carried an estimated median disease-free survival (DFS) and overall survival (OS) of 6 and 9 months, respectively, without consolidative hematopoietic stem cell transplantation (HSCT) (1, 2). Allogeneic HSCT was considered the only curative option for this group, with the earliest reports describing OS rates of about 30% (3, 4). A groundbreaking discovery was the development of rational targeted tyrosine kinase inhibitors (TKIs) as therapy for hematologic malignancies (5). Addition of breakpoint cluster region–Abelson kinase (BCR-ABL) inhibitors, such as imatinib and dasatinib, to standard cytotoxic therapy has greatly improved upfront treatment and early response, enabling increased eligibility of adults for subsequent curative HSCT (6, 7). In addition, intensive chemotherapeutic regimens with TKIs allow some patients to attain extended DFS without consolidative allogeneic transplantation (8, 9). However, many patients are unable to tolerate the intensive combination of chemotherapeutic regimens typically used to treat this disease, and relapse either before or after HSCT is challenging to treat, with a median survival of only 4 to 5 months (10). A simplified treatment approach for coping with cytotoxic chemotherapy used 6 days of pretreatment with high-dose steroids and then 21 days of induction therapy with dasatinib and continuation of high-dose steroids (11). After 21 days of treatment, steroids were rapidly tapered off, and patients remained on dasatinib monotherapy until the time of either allogeneic HSCT or disease progression. All patients achieved a complete hematologic response, with 22% achieving a serum BCR-ABL transcript level lower than 1 × 10−3. On the basis of these encouraging results, many adult patients with Ph+ ALL are treated with this regimen, followed by dasatinib monotherapy. Unfortunately, without consolidative allogeneic HSCT, most patients will experience a relapse secondary to resistance within an average of 6 months (12).

Resistance to TKIs can occur through development of/selection for ABL kinase mutations or increased dependence on parallel pathways that do not involve ABL signaling. The end results are enhanced survival and avoidance of apoptosis via up-regulation of the antiapoptotic members of the B cell lymphoma 2 (BCL-2) family of proteins (13, 14). The BCL-2 family of proteins consists of the antiapoptotic members BCL-2, BCL-xL, and MCL-1 (myeloid cell leukemia 1), as well as the prodeath members BAX, BAD, BAK, NOXA, and BIM. Overexpression of the antiapoptotic molecule BCL-2 and suppression of the prodeath molecule BIM or BAX have been shown to be major contributors to the pathogenesis of many lymphoid malignancies, making antagonism of the antiapoptotic BCL-2 proteins a compelling target for antitumor therapy (15). Although previous studies have suggested that Ph+ALL has a relatively low expression of BCL-2 (16), the dependence of Ph+ALL on BCL-2 has been previously demonstrated by RNA interference targeting of BCL-2, which strongly induced apoptosis in Ph+ALL cell lines (17). In addition, mice xenografted with Ph+ALL showed an impressive response to treatment with the BCL inhibitor ABT-737 when used as a single agent (17).

Recently, a selective inhibitor of BCL-2, ABT-199 (venetoclax), has shown impressive activity against hematologic malignancies, including chronic lymphocytic leukemia (18, 19), acute myeloid leukemia (AML) (20), non-Hodgkin’s lymphoma (21), and subtypes of ALL such as those with mixed lineage leukemia (MLL) rearrangements (22). Because venetoclax selectively inhibits BCL-2 but not MCL-1, malignancies that
are particularly susceptible to this drug are those that display high BCL-2 expression or BCL-2 addiction and a low BCL-2/MCL-1 ratio, as assessed by transcript quantification (23). In contrast, a recent mouse model for Ph+ALL showed the importance of aberrant MCL-1 expression for leukemogenesis (24). Selective inhibition of MCL-1 expression or activity had a potential therapeutic effect. Therefore, the clinical importance of the inhibition of BCL-2 versus MCL-1 in Ph+ALL remains unknown. We sought to clarify the activity of venetoclax in Ph+ALL and to test combination therapy using TKIs in the hope of identifying a better combination therapy for this aggressive subtype of ALL.

RESULTS

Leukemic cells that are sensitive to venetoclax express a higher BCL-2/MCL-1 ratio

We first sought to determine the characteristics of leukemia cells that would respond to inhibition of the BCL-2 family of proteins with venetoclax. We chose three representative cell lines that are known to be kinase-dependent: K562 cells (a chronic myeloid leukemia (CML) cell line dependent on BCR-ABL1 activity), RCH-ACV (ALL with TCF3-PBX1 dependent on the activity of the pre-B cell receptor), and SUPB15 (an ALL cell line dependent on BCR-ABL1 activity). SUPB15 cells showed exquisite sensitivity to venetoclax, with a median inhibitory concentration (IC50) between 1 and 10 nM (Fig. 1A). In contrast, RCH and K562 cells showed minimal sensitivity, suggesting little dependence on BCL-2. Similar to SUPB15, primary Ph+ALL patient samples 12-149 and 12-225 showed sensitivity to venetoclax and dasatinib (Fig. 1B). In agreement with previous studies showing the importance of the BCL-2/MCL-1 ratio for sensitivity to BCL-2 inhibitors (25-28), immunoblot analysis verified that the Ph+ALL cells (SUPB15, 12-149, and 12-225) had a higher BCL-2/MCL-1 ratio than the TCF3-PBX1 cells (RCH-ACV, 07-112, and 11-064) (Fig. 1C). Moreover, reduction of MCL-1 expression in RCH cells with MCL-1–specific small interfering RNA (siRNA) increased sensitivity to venetoclax (Fig. 1D).

The addition of venetoclax to dasatinib results in enhanced effects in vitro

Although multiple ABL kinase inhibitors are used in the treatment of BCR-ABL1–dependent disease, dasatinib is the current backbone for the treatment of adult Ph+ALL. Therefore, we chose to first evaluate the combination of dasatinib and venetoclax. The addition of venetoclax to dasatinib resulted in a stepwise reduction in the IC50 of dasatinib, with increasing doses of venetoclax producing larger reductions in the IC50 (Fig. 2A). All combinations of dasatinib and venetoclax tested were calculated to be synergistic, with combination index (CI) values ranging between 0.07 and 0.65 (values < 1 were considered synergistic) (Fig. 2B and table S1). This synergy was conserved in the primary Ph+ALL sample 12-149 (Fig. 2C). We additionally found synergy between venetoclax and cytarabine, dexamethasone, doxorubicin, and vincristine, suggesting that venetoclax could potentially be used with standard chemotherapy in addition to combination with dasatinib (fig. S1).

Fig. 1. TKI-sensitive leukemia cells with higher BCL-2/MCL-1 ratio respond to venetoclax. (A) Dose-response curves of three leukemia cell lines known to be kinase-driven. Cells were treated with increasing concentrations of venetoclax for 72 hours, then assayed by MTS as an indirect measure of viability. Absorbance at 490 nm was measured and normalized to untreated cells, defined as 100%. RCH, blue triangles; K562, blue squares; SUPB15, red diamonds. Each point represents the mean ± SEM (n = 3). (B) Drug sensitivity of Ph+ALL samples 12-149 and 12-225 to increasing concentrations of dasatinib (black to gray bars) and venetoclax (shades of red bars). Cells were treated with increasing concentrations of each drug over 48 to 72 hours, then assayed for viability using MTS and normalized to no-drug control. Each bar represents the mean ± SD (n = 3, with the exception of 12-225 (n = 1)). (C) Immunoblot analysis of leukemia cells. Samples were blotted for MCL-1, BCL-2, and tubulin. Samples 07-112 and 11-064 are primary TCF3-PBX1 samples. The immunoblot represents three independent experiments that were subsequently quantified for the BCL-2/MCL-1 ratio normalized to SUPB15. Each bar represents the mean ± SD (n = 3). The graph on the right shows the average ratio for Ph+ cells compared to the average ratio for TCF3-PBX1 (*P = 0.0193, unpaired two-tailed t test). (D) Dose response of RCH cells to venetoclax with MCL-1 siRNA treatment. RCH cells were treated with MCL-1 (red triangles) or nonspecific (NS) (black diamonds) siRNA, then treated with increasing concentrations of venetoclax for 72 hours and assayed by MTS. Results were normalized to no drug in the NS siRNA control. Each point represents the mean ± SEM (n = 3). A portion of the no-drug–treated cells were harvested 24 hours after siRNA treatment, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and blotted for MCL-1 and tubulin (inset).
We then evaluated the effect of the combination of dasatinib and venetoclax on apoptosis. Whereas dasatinib alone was able to increase annexin V staining to some extent, venetoclax was a stronger inducer of apoptosis at all time points assessed (Fig. 2D). Furthermore, the combination of dasatinib and venetoclax resulted in a greater degree of apoptosis than either agent alone at all time points assessed (Fig. 2D).

Inhibition of BCR-ABL1 activity by dasatinib decreases phosphorylation (p-) CRKL and subsequently increases apoptosis (13, 14, 29). Meanwhile, inhibition of BCL-2 by venetoclax enhances apoptosis by activating the caspase pathway through caspase 9. To test whether the combination increases caspase-dependent apoptosis, we quantified cleaved poly(ADP-ribose) polymerase (PARP) and caspase 9 at 4 and 24 hours in cells treated with dasatinib, venetoclax, or their combination. By 4 hours, cells treated with dasatinib or the combination showed a marked inhibition of phospho-CRKL with no effect by venetoclax, whereas venetoclax and the combination showed an increase in cleaved PARP and cleaved caspase 9. By 24 hours, the combination treatment showed increased cleaved PARP and cleaved caspase 9 compared to individual drugs, demonstrating enhanced apoptosis through caspase activity (Fig. 2E).

The multikinase inhibitors ponatinib and dasatinib show enhanced response with venetoclax through inhibition of LYN activity in Ph‘ALL

We next sought to determine whether synergy was present across all of the other TKIs now available for treatment of Ph‘ALL. Because different TKIs have different selectivities for inhibition of classes of kinases, this variability between drugs could identify other pathways important for viability (30, 31). Although testing of all of the TKIs (imatinib, nilotinib, dasatinib, and ponatinib) with venetoclax showed some degree of synergy, the most robust synergy was seen with dasatinib or ponatinib (fig. S2 and table S1).

We then compared the TKIs in terms of their ability to enhance apoptosis, as assessed by annexin V staining. By 4 hours, venetoclax already had notable effects on apoptosis, whereas none of the kinase inhibitors showed much activity above background (Fig. 3A). However, both dasatinib and ponatinib enhanced the apoptosis triggered by venetoclax, whereas imatinib and nilotinib did not (Fig. 3A). We then tested what effects the different kinase inhibitors had on the BCL-2 family members as single agents. SUPB15 cells treated with drugs for 24 hours showed no change in the expression of the proapoptotic
Dual inhibition of ABL and LYN in Ph+ALL enhances the effectiveness of treatment with TKI and venetoclax. (A) TKIs’ effects on apoptosis when combined with venetoclax. SUPB15 cells were treated with 5000 nM imatinib, 500 nM nilotinib, 50 nM dasatinib, or 50 nM ponatinib, alone (black bars) or in combination with 50 nM venetoclax (gray bars), for 4 hours. The samples were then stained with annexin V and analyzed. Baseline untreated annexin V staining was subtracted from each bar. Each bar represents the mean ± SD (n = 3). (B) Effect of TKIs on the expression of the proapoptotic BCL-2 family member BIM and the antiapoptotic members BCL-1 and MCL-1. Cells were treated with TKIs as in (A) for 24 hours, then immunoblotted for BIM, BCL-2, MCL-1, and tubulin. (C) Effect of the TKIs and venetoclax on other proapoptotic molecules. Cells were treated with drugs for 24 hours, then immunoblotted for BAX, BID, PUMA, NOXA, cleaved PARP, and tubulin. (D) Effect of the TKIs and venetoclax on kinase activity. SUPB15 cells were treated with the indicated drugs for 24 hours, then immunoblotted for BAX, BID, PUMA, NOXA, cleaved PARP, and tubulin. (E) Viability of SUPB15 cells after knockdown with ABL1, LYN, or BTK siRNA. Cells were treated with NS (black bar), BTK (red bar), LYN (green bar), or ABL (blue bar) siRNA for 72 hours and assessed with MTS. Results were normalized to cells treated with NS siRNA. Each bar represents the mean ± SD (n = 3). (F) Apoptosis was assessed in SUPB15 cells treated with siRNA for NS, LYN, ABL1, or ABL1 + LYN, alone or in combination with 50 nM venetoclax. Twenty-four hours after electroporation, treated cells were split to receive no treatment (black bars) or 50 nM venetoclax (gray bars) for 4 hours and assessed for annexin V staining. Each bar represents the mean ± SD (n = 3). A portion of the sample was also harvested, and the cell lysate was immunoblotted for ABL1 or LYN (inset).

![Graph showing the effects of TKIs and venetoclax on apoptosis and kinase activity.](image)

Because dasatinib is also known to inhibit BTK and LYN, which play important roles in B cell malignancies, we assessed their importance in Ph+ALL. Cells incubated with imatinib, nilotinib, dasatinib, or ponatinib were assessed for phosphorylation of CRKL, LYN, and BTK (Fig. 3D). Although all TKIs decreased phospho-CRKL, only dasatinib and ponatinib decreased phospho-LYN. In addition, dasatinib inhibited the phosphorylation of BTK. To determine whether inhibition of either LYN or BTK contributed to enhanced cell death, we treated cells with siRNA targeting ABL, LYN, or BTK. Only ABL and LYN knockdown showed decreased viability overall, as compared to control cells, in terms of overall viability and percentage of apoptosis (Fig. 3, E and F). Combined knockdown of LYN and ABL showed even greater apoptosis as compared to knockdown of either kinase alone (Fig. 3F). When cells treated with siRNA to LYN and ABL were exposed to venetoclax for 24 hours, they exhibited enhanced apoptosis. Therefore, these findings suggest that inhibiting both ABL and LYN through dasatinib or ponatinib enhances synergy with venetoclax.

Treatment of xenografted animals with the combination of venetoclax and dasatinib is tolerable and shows enhanced response in vivo

As a model for Ph+ALL, we injected a cohort of NOD/SCID/IL-2Rγnull (nonobese diabetic/severe combined immunodeficient/interleukin-2 receptor gamma null) (NSG) mice with a primary Ph+ALL sample (12-225). Previous studies have used dasatinib (40 to 100 mg/kg) (32) and venetoclax (50 to 100 mg/kg) (33) as both tolerable and therapeutic doses in murine models. To test the effect of combination therapy, we treated animals with dasatinib (40 mg/kg) and venetoclax (25 mg/kg) daily for 5 days per week for 4 weeks after they exhibited 5 to 10% human CD45 cells circulating in the peripheral blood (Fig. 4A). During this time,
each animal was assessed daily for its general well-being, including lethargy, abnormal locomotion, ruffled fur, arched back, or overt weight loss, following the institutional animal care and use policy. The treated group tolerated the therapy well with no change in physical appearance, whereas untreated mice quickly became moribund after achieving peripheral blood chimerism greater than 10%. Mice treated with the combination showed a reduction of their peripheral blood human CD45 (hCD45) within 4 days and remained alive during the 4 weeks of treatment (Fig. 4A). Spleen size at the time of harvest was significantly smaller after 4 weeks of treatment as compared to the spleen size of control animals (*P = 0.0158) (Fig. 4A).

These initial studies suggested that the combination was tolerable and therapeutic. To test whether the combination showed synergy in vivo, we injected NSG mice with mononuclear cells from a different patient sample with Ph⁺ ALL (12-149). Because the CI values predicted synergy at doses 10-fold lower than that for single-agent treatment, we chose to use one-tenth of the doses used in previous murine studies [dasatinib (5 mg/kg) and venetoclax (5 mg/kg)] (33–35). The control group and the single-agent venetoclax group engrafted within 60 days, but only two of five mice in the dasatinib group and none in the combination group engrafted within 90 days of treatment (Fig. 4B). At the time of euthanasia, the spleens and bone marrows were assessed for disease burden. Animals treated with the combination of dasatinib and venetoclax were found to have significantly smaller spleen weights than those in the control group or either of the single-agent treatment groups (**P = 0.0018) (Fig. 4C). Histochemical examination of tissue showed that bone marrow from the control group and from venetoclax-treated animals was completely effaced by leukemic blasts. In contrast, the dasatinib monotherapy group had less effacement of tissue by leukemic cells, and combination therapy had even less involvement (Fig. 4D). Together, our xenograft model supports the concept of combination therapy, as compared to single-agent treatment, for decreasing disease burden.

**Dasatinib and ponatinib prevent an increase in MCL-1 through inhibition of LYN and phosphorylation of STAT5**

One possible advantage to combination therapy is the potential to preempt the emergence of resistance. Because up-regulation of MCL-1 is a known mechanism of resistance to venetoclax (24, 26, 36), we wanted to quantify MCL-1 in the leukemic cells from animals that received single-agent venetoclax. Similar to SUPB15 cells exposed to venetoclax, spleen cells harvested from 12-149 mice treated with venetoclax showed increased MCL-1 as compared to control, dasatinib, or combination treatment (Fig. 5A).

To determine whether other TKIs prevented this increase in MCL-1, we treated SUPB15 cells with a combination of TKI plus venetoclax. Only treatment with dasatinib or ponatinib prevented an increase in MCL-1, suggesting a link to LYN activity (Fig. 5B). In addition, as with single-agent treatment, BIM was increased only in venetoclax combinations including dasatinib and ponatinib. Finally, treatment with LYN siRNA inhibited the increase in MCL-1 seen with venetoclax treatment (Fig. 5C).

To explore potential non-ABL kinase targets, we performed a phospho-proteome array analysis comparing cells treated with imatinib or dasatinib to cells treated with control (fig. S3). Although several substrates showed different changes in phosphorylation, we found that, as compared to control and imatinib, dasatinib treatment was more effective at inhibiting...
STAT5a/b (fig. S3). Previous studies have shown that STAT5 activation is important in the regulation of MCL-1 expression in pro-B lymphocytes (37). Furthermore, STAT5 phosphorylation is dependent on BCR-ABL1 activity in CML (38). However, we found that treatment with the ABL inhibitors imatinib and nilotinib had minimal effect on STAT5 phosphorylation compared to treatment with dasatinib and ponatinib (Fig. 5D and fig. S3). In addition, treatment with the LYN selective inhibitor saracatinib inhibited phosphorylation of STAT5 (Fig. 5D), further supporting LYN activity linked to STAT5 phosphorylation. To assess for a potential interaction between LYN and STAT5, we subjected the cell lysate from SUPB15 to immunoprecipitation with an anti-LYN antibody and immunoblotted it for STAT5. As compared to the isotype control, STAT5 was enriched in association with LYN (Fig. 5E). These findings suggest that inhibition of LYN activity by dasatinib results in inhibition of STAT5 phosphorylation, which in turn prevents the increase in MCL-1. Together, these results suggest that LYN activity may be important for regulating BIM and MCL-1 through STAT5.

DISCUSSION

The advent of TKIs has improved early response to single-agent treatment and combination chemotherapy. Unfortunately, a major limitation to single-agent targeted therapies is resistance through the development of mutations that render the cells insensitive to the targeted agent, as well as changes in the cells’ dependence on alternative survival pathways. Dependence on several different pathways in imatinib-resistant CML and Ph+ALL cell lines has been described, including the following: the SRC/LYN pathway (39–42), the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway (43), the PI3K (phosphatidylinositol 3-kinase)/AKT pathway (44, 45), and the RAS/RAF/MEK (mitogen-activated protein kinase/extracellular signal–regulated kinase) pathway (46, 47). However, the relative contributions of these pathways to resistance in patients with Ph+ALL are neither well described nor screened for routinely, complicating the selection of optimal treatment. In addition, combining multiple kinase inhibitors has the potential to be more effective but may have more toxicities (48).

Avoidance of apoptosis is a hallmark of all of the survival pathways, and overexpression of BCL-2 is a major contributor to the pathogenesis of many lymphoid malignancies (49). With the development of inhibitors of antiapoptotic BCL-2 proteins, dual targeting of a kinase pathway, in combination with inhibition of apoptosis, has been an area of intense investigation. The addition of the pan-BCL-2 family inhibitor ABT-737 to a MEK inhibitor with or without a PI3K inhibitor was synergistic in both lung and pancreatic cancer cell lines, particularly those that harbored a KRAS (Kirsten Ras) mutation. This synergy was mediated through the combined inhibition of BCL-xL by ABT-737 and the up-regulation of BIM by a MEK inhibitor (50). In AML cell lines, the combination of a MEK inhibitor and the BCL-2 inhibitor ABT-737 was highly effective, although neither drug was capable of killing AML cells alone. Treatment with ABT-737 increased the amount of MCL-1, but this effect was abrogated by combination with a MEK inhibitor (51). In CML cell lines, single-agent ABT-737 had little effect on cells, but it restored sensitivity to imatinib in imatinib-resistant CML cell lines and enhanced cell killing with a second-generation ABL kinase inhibitor, INNO-406. The ABL kinase inhibitors resulted in up-regulation of BIM, and ABT-737 inhibited BCL-xL and BCL-2 (52). In each of these models, the actions of combined kinase inhibition and BCL-2 inhibition seemed to be mediated by tipping the balance of antiapoptotic and proapoptotic proteins. Our studies further validate this concept of combining TKIs with BCL-2 inhibitors to improve the efficacy of each drug. Although previous studies have shown activity of the BCL-2 inhibitors in Ph+ALL (24), our study additionally shows synergy with TKIs.

Fig. 5. The amount of MCL-1 is regulated by LYN and STAT5 in Ph+ALL. (A) Amounts of MCL-1 and BCL-2 after drug treatment. SUPB15 and mononuclear cells from the spleen of engrafted mice with 12-149x were treated with 50 nM venetoclax, 50 nM dasatinib, or the combination for 24 hours, then harvested to quantify MCL-1, BCL-2, and tubulin. (B) The effects of combination treatment with TKIs and venetoclax on MCL-1 and BIM. SUPB15 cells were treated with 50 nM venetoclax alone or combined with 5000 nM imatinib, 500 nM nilotinib, 50 nM dasatinib, or 50 nM ponatinib for 16 hours, harvested, and immunoblotted for MCL-1, BIM, and tubulin. (C) The effects of LYN siRNA treatment on MCL-1. Twenty-four hours after electroporation of SUPB15 cells with NS or LYN siRNA, the cells were treated with 50 nM venetoclax or vehicle for an additional 24 hours, then immunoblotted for LYN, MCL-1, and tubulin. (D) STAT5 phosphorylation after treatment with TKIs. SUPB15 cells were treated with 5000 nM imatinib, 500 nM nilotinib, 50 nM dasatinib, or 500 nM ponatinib, or 500 nM saracatinib for 16 hours, then immunoblotted for phospho-STAT5, total STAT5, and tubulin. (E) Immunoprecipitation with anti-LYN antibody. Cell lysate from SUPB15 cells was incubated with anti-LYN antibody or rabbit immunoglobulin G (IgG) overnight at 4°C, Antibody was recovered using Protein G Sepharose and immunoblotted for STAT5 and LYN.
from its inhibition of BCL-xL. (53). After this, further clinical trials of navitoclax in pediatric ALL were abandoned. Meanwhile, a recent study further supported the use of venetoclax in subsets of ALL (22). Benito et al. (22) found that MLL-rearranged leukemias increase BCL-2 expression through DOT1L (disruptor of telomeric silencing 1-like)—mediated H3K79me2/3. Within their data set, MLL-rearranged ALL showed marked overexpression of BCL-2 and lower MCL-1. Although the expression of BCL-2 in their Ph’ALL subset was not markedly elevated, we were able to confirm that SUPB15 and two primary Ph’ALL samples were sensitive to BCL-2-specific inhibition with higher BCL-2/MCL-1 ratios. Furthermore, a panel of pre-B ALL samples xenografted into mice and tested for response to ABT-737 showed that ABT-737 potentiated the response of the chemotherapeutic agents vincristine, L-asparaginase, and etoposide (54). In T ALL cell lines, venetoclax synergistically potentiated the anti-leukemic effects of doxorubicin, L-asparaginase, and dexamethasone (33).

The importance of SRC family kinases, such as LYN, FGR, and LCK, in Ph’ALL has been previously noted (39). However, beyond its direct effects on viability, LYN activity seems to play an important role in the control of MCL-1, likely through STAT5 phosphorylation. STAT5 activation is important in the regulation of MCL-1 expression in pro-B lymphocytes (37). In addition, STAT5 plays a role in the stabilization of MCL-1 protein content in T cells, which can be inhibited by a JAK-STAT inhibitor (55). We found that in Ph’ALL, phosphorylation of STAT5 was preferentially inhibited by dasatinib and ponatinib, as well as by the SRC family inhibitor saracatinib, and that STAT5 coprecipitates with LYN, suggesting the possibility of a direct complex.

Previous studies have also evaluated the role of MCL-1 in the development of venetoclax resistance and found that venetoclax can cause rapid up-regulation of MCL-1 (24, 36). This increase was found to be secondary to stabilization of MCL-1 protein rather than an increase in MCL-1 transcription. Other studies have shown that the characteristics of venetoclax-resistant cells involve not only the up-regulation of MCL-1 but also the changes in both its stability and its ability to interact with BIM and NOXA (26, 36). Although our studies suggest that LYN/STAT5 activity can be important for MCL-1 regulation, its role in the development of long-term venetoclax resistance remains to be seen.

Our study has several limitations that warrant discussion. Although two primary Ph’ALL samples were included and used for the xenograft studies, most biochemical studies were based on SUPB15 cells as the model for Ph’ALL. Whether these findings can be generalized to all patients with Ph’ALL or to a subset of patients remains unknown. In addition, the mechanism of STAT5 phosphorylation through LYN has not been addressed in our studies. LYN activity may activate other downstream targets to eventually phosphorylate STAT5 or it may directly phosphorylate STAT5 as previously shown (56).

In conclusion, our preclinical results demonstrate that inhibition of ABL and LYN, in combination with BCL-2, is highly synergistic in vitro by enhancing apoptosis. This effect is mediated by an increase in the proapoptotic molecule BIM and prevention of increase in the antiapoptotic molecule MCL-1, likely through inhibition of STAT5. These results lay the foundation for the testing of this combination in patients with Ph’ALL with the goal of improving treatment.

MATERIALS AND METHODS

Study design

The primary objective of these studies was to investigate the use of BCL-2 inhibition, in combination with kinase inhibition, in Ph’ALL. In vitro studies using a human-derived leukemia cell line and primary patient samples explored the mechanism of synergy between TKI and BCL-2 inhibition through standard biochemical techniques. In vivo studies included cohorts of murine xenograft models based on primary patient samples treated with drugs in a nonblinded manner.

Chemicals and reagents

Fetal bovine serum (FBS) was obtained from HyClone (Thermo Fisher Scientific). All other tissue culture reagents were obtained from Invitrogen. Viability assays were performed with CellTiter 96 AQeues One Solution Cell Proliferation Assay (MTS; Promega) and quantified using Gen5 (BioTek). Annexin V assays were performed using Guava Nexin (EDP Millipore) and analyzed with Guava ViaCount software. Venetoclax was purchased from Selleckchem and solubilized in dimethyl sulfoxide (DMSO) at 10 mM stock. Dasatinib was purchased from LC Laboratories and solubilized in DMSO at 10 mM stock. For murine treatment, dasatinib was dissolved in 80 mM sodium citrate (pH 3.1) (dasatinib vehicle) at 10 mg/ml, and venetoclax was dissolved in 60% phospholipid glycol, 30% polyethylene glycol 400, and 10% ethanol (venetoclax vehicle) at 5 mg/ml. Graphical and statistical data were generated using GraphPad Prism or CalcuSyn.

Cell lines and tissue culture

SUPB15 [American Type Culture Collection (ATCC)] is a pediatric ALL cell line from a patient with recurrent disease carrying the BCR-ABL1 t(9;22) translocation. K562 (ATCC) is a CML cell line that also carries the BCR-ABL1 t(9;22) translocation. RCH-ACV (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) is a pediatric ALL cell line from a patient with recurrent disease carrying the TCF3-PBX1 t(1;19) translocation. SUPB15 was maintained in RPMI with 20% FBS, 4 mM glutamine, and 1% penicillin and streptomycin. K562 was maintained in RPMI with 10% FBS, 1% penicillin and streptomycin.

No human subjects were directly involved in the research. Biological samples were obtained with written informed consent. Procurement of patient samples was approved by the Institutional Review Board of Oregon Health and Science University (IRB no. 4422).

siRNA treatment

Cells (1 million cells per treatment) were incubated with 40 µM NS, ABL1, LYN, or BTK siRNA (Dharmacon) in siPORT siRNA Electroporation Buffer (Life Technologies) then electroporated using GenePulser Xcell (Bio-Rad).

Immunoblot

Cell pellets were lysed with cell lysis buffer (Cell Signaling) with Complete Mini (Roche), aprotinin, phosphatase inhibitor cocktail 2 (Sigma), and 1 mM phenylmethylsulfonyl fluoride. Fifty micrograms of total protein was separated per well on a 4 to 15% or 4 to 20% SDS-PAGE, transferred onto Immobilon-FL, blocked with AquaBlock/ELA/WB (EastCoast Bio) for 1 hour, and incubated with primary antibody in AquaBlock/0.1% Tween 20 overnight at 4°C. Blots were probed with the following antibodies (Cell Signaling): anti-MCL-1, anti–BCL-2, anti–BCL-xL, anti-LYN, anti-ABL1, anti-BTK, anti–phospho-LYN, anti–phospho-CRKl, anti–phospho-BTK, anti-BIM, anti-BAX, anti-BAK, anti-BID, anti-PUMA, anti–capsase 9, anti–cleaved PARP, and anti-tubulin. Secondary fluorescent antibodies (anti-rabbit or anti-mouse; Molecular Probes) were used and detected with Odyssey (LI-COR), or secondary antibodies linked with horseradish peroxidase (Promega) were detected by enhanced chemiluminescence (Bio-Rad), and the
In vivo treatment of xenografts with dasatinib and venetoclax

Xenografts of primary patient samples were established as previously described (35, 57–60). All animal studies were conducted with approval from the Oregon Health and Science University's Institutional Animal Care and Use Committee (IACUC, protocol no. 2358). Veterinary care was provided through the Oregon Health and Science University Department of Comparative Medicine. NSG mice were sublethally irradiated with 200 centigrays at age 6 weeks and, 24 hours later, were anesthetized with isoflurane and injected via the tail vein with 1 million cells from frozen patient samples. Mice were then provided with ciproflucacin (0.25 mg/ml) containing water for 4 weeks during recovery. Peripheral blood was monitored by flow cytometry for human engraftment using antibodies against hCD45-FITC (fluorescein isothiocyanate) (Miltenyi Biotec) versus murine CD45 (mCD45)–PerCP-Cy5.5 (BD Pharmingen) weekly starting on week 3. Flow cytometry data were analyzed using a fluorescence-activated cell sorter (FACSAria Cy5.5 (BD Pharmingen) weekly starting on week 3. Flow cytometry data were analyzed using a fluorescence-activated cell sorter (FACSAria II).

Fig. S1. Dose-response curves for SUPB15 cells treated with the combination of cytotoxic chemotherapy with venetoclax.

Table S1. Combination indices of TKIs and cytotoxic chemotherapy with venetoclax.

Fig. S2. Dose-response curves for SUPB15 cells treated with the combination of TN and venetoclax.

Table S3. Combination indices of TKIs and cytotoxic chemotherapy with venetoclax.

Statistical analysis

Data were analyzed by two-tailed Student’s t test. Differences were considered statistically significant at P < 0.05.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/8/354/354ra114/DC1

REFERENCES AND NOTES


RESEARCH ARTICLE


254825) inhibits Stat5 signaling associated with apoptosis in chronic myelogenous leuke-

blastic leukemia.

ABT-199 mediated inhibition of BCL-2 as a novel therapeutic strategy in T-cell acute lympho-

blastic leukemia.

BCL-2-specific antagonist ABT-199.


Synergistic induction of apoptosis in high-risk DLBCL by BCL2 inhibition

with pharmacologic loss of MCL1. Leukemia 29, 1702–1712


Mol. Cancer Ther.


B. Koss, J. Morrison, R. M. Percivalle, H. Singh, J. E. Rehg, R. T. Williams, J. T. Opferman, Requirement for antitumor MCL-1 in the survival of BCR-ABL B-lineage acute lympho-


43. A. K. Samanta, S. N. Chakraborty, Y. Wang, H. Kantarjian, X. Sun, J. Hood, D. Perrotti, A. Roberts, Targeting BCL2 for the treatment of lymphoid malig-


larity in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interac-


55. A. R. Shenoy, S. Kirschnek, G. Haeker, IL-15 regulates Bcl-2 family members Bim and Mcl-

larity in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interac-


55. A. R. Shenoy, S. Kirschnek, G. Haeker, IL-15 regulates Bcl-2 family members Bim and Mcl-

larity in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interac-


55. A. R. Shenoy, S. Kirschnek, G. Haeker, IL-15 regulates Bcl-2 family members Bim and Mcl-

larity in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interac-


55. A. R. Shenoy, S. Kirschnek, G. Haeker, IL-15 regulates Bcl-2 family members Bim and Mcl-

larity in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interac-

a faster and more efficient disease compared to other NOD/scid-related strains. Int. J. Cancer 123, 2222–2227 (2008).


Acknowledgments: We thank B. Garcia for her expertise in flow cytometry. Funding: This work was supported in part by the Leukemia & Lymphoma Society (Leukemia & Lymphoma Society Specialized Center of Research grant 7005-11 and Leukemia & Lymphoma Society Beat AML) and the Newman’s Own Foundation. B.H.C. was further supported by grants from the Hyundai Hope on Wheels and the St. Baldrick’s Foundation. J.W.T. was supported by the V Foundation for Cancer Research, the Gabrielle’s Angel Foundation for Cancer Research, and the National Cancer Institute (5R00CA151457-04 and 1R01CA183947-01). B.J.D. was supported by the Howard Hughes Medical Institute. Author contributions: J.T.L. conceived, designed, and performed the experiments, and co-wrote the manuscript. J.S.J.R. performed the xenograft experiments. C.A.E. assisted in the interpretation of patient sample studies. E.T. assisted in the design of the studies. B.H.-L. assisted in the design of the studies. S.E.S. assisted in the design of the studies. M.L. also assisted in the design of the studies. B.J.D. assisted in the design of the studies. J.W.T. assisted in the design of, directed, and co-wrote the manuscript. B.H.C. conceived, directed, and assisted in the experiments, and co-wrote the manuscript.

Competing interests: J.W.T. receives research support from Agios Pharmaceuticals, Array BioPharma, Aptose Biosciences, AstraZeneca, Constellation Pharmaceuticals, Genentech, Incyte, Janssen Research & Development, Seattle Genetics, and Takeda Pharmaceuticals, and is a consultant for Leap Oncology. S.E.S. receives research support from Acerta Pharma, Genentech, Gilead Sciences, Bristol Meyers Squibb, and Janssen Research & Development. B.J.D. serves as a consultant to and/or on the scientific advisory boards of Aptose Biosciences, Beta Cat, Blueprint Medicines, CTI Biopharma, Gilead Sciences, GRAIL, MED-C, MolecularMD, and Third Coast Therapeutics. The other authors declare that they have no competing interests.

Submitted 26 February 2016
Accepted 29 July 2016
Published 31 August 2016
10.1126/scitranslmed.aaf5309

Targeting BCL-2 and ABL/LYN in Philadelphia chromosome–positive acute lymphoblastic leukemia

Jessica T. Leonard, Joelle S. J. Rowley, Christopher A. Eide, Elie Traer, Brandon Hayes-Lattin, Marc Loriaux, Stephen E. Spurgeon, Brian J. Druker, Jeffrey W. Tyner and Bill H. Chang

DOI: 10.1126/scitranslmed.aaf5309

Showing ALL that resistance is futile

Philadelphia chromosome–positive acute lymphoblastic leukemia (Ph+ALL) carries the BCR-ABL translocation; however, despite the existence of drugs that target this translocation, Ph+ALL remains very difficult to treat, and single-drug treatment is usually ineffective. Leonard et al. showed that venetoclax, an apoptosis-promoting drug, is effective in treating this disease and that dasatinib, an inhibitor of BCR-ABL, blocks a common mechanism of resistance to venetoclax. As a result, these two drugs work together synergistically in vitro and in preclinical models, offering a promising approach toward the rational treatment of this disease.

ARTICLE TOOLS
http://stm.sciencemag.org/content/8/354/354ra114

SUPPLEMENTARY MATERIALS
http://stm.sciencemag.org/content/suppl/2016/08/29/8.354.354ra114.DC1

RELATED CONTENT
http://stm.sciencemag.org/content/scitransmed/7/279/279ra40.full
http://stm.sciencemag.org/content/scitransmed/6/252/252ra121.full
http://stm.sciencemag.org/content/scitransmed/8/339/339ra69.full
http://stm.sciencemag.org/content/scitransmed/8/339/339ra70.full
http://stm.sciencemag.org/content/scitransmed/8/355/355ra117.full
http://stm.sciencemag.org/content/sigtrans/9/456/ra116.full
http://stm.sciencemag.org/content/scitransmed/9/401/eaam7049.full
http://stm.sciencemag.org/content/scitransmed/9/417/eaan8723.full
http://stm.sciencemag.org/content/scitransmed/10/427/eaan8735.full
http://stm.sciencemag.org/content/scitransmed/10/445/eaan1240.full

REFERENCES
This article cites 58 articles, 18 of which you can access for free
http://stm.sciencemag.org/content/8/354/354ra114#BIBL

PERMISSIONS
http://www.sciencemag.org/help/reprints-and-permissions

Use of this article is subject to the Terms of Service