NOTCH1 Represses MCL-1 Levels in GSI-resistant T-ALL, Making them Susceptible to ABT-263

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Abstract

Purpose: Effective targeted therapies are lacking for refractory and relapsed T-cell acute lymphoblastic leukemia (T-ALL). Suppression of the NOTCH pathway using gamma-secretase inhibitors (GSI) is toxic and clinically not effective. The goal of this study was to identify alternative therapeutic strategies for T-ALL.

Experimental Design: We performed a comprehensive analysis of our high-throughput drug screen across hundreds of human cell lines including 15 T-ALL models. We validated and further studied the top hit, navitoclax (ABT-263). We used multiple human T-ALL cell lines as well as primary patient samples, and performed both in vitro experiments and in vivo studies on patient-derived xenograft models.

Results: We found that T-ALL are hypersensitive to navitoclax, an inhibitor of BCL2 family of antiapoptotic proteins. Importantly, GSI-resistant T-ALL are also susceptible to navitoclax. Sensitivity to navitoclax is due to low levels of MCL-1 in T-ALL. We identify an unsuspected regulation of mTORC1 by the NOTCH pathway, resulting in increased MCL-1 upon GSI treatment. Finally, we show that pharmacologic inhibition of mTORC1 lowers MCL-1 levels and further sensitizes cells to navitoclax in vitro and leads to tumor regressions in vivo.

Conclusions: Our results support the development of navitoclax, as single agent and in combination with mTOR inhibitors, as a new therapeutic strategy for T-ALL, including in the setting of GSI resistance.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive blood cancer that accounts for 15% of pediatric and 25% of adult ALL cases (1). Although significant advances in chemotherapy regimens have led to high rates of complete remission, close to 50% of adults and 25% of pediatric cases relapse within the first two years (2). Unfortunately, for patients with relapsed or refractory leukemia, the prognosis remains poor, emphasizing the need to develop more effective antileukemic targeted therapies.

Although the detailed molecular mechanisms that account for the aggressive nature and poor therapeutic response of T-ALL remain to be fully elucidated, studies have shown that mutations activating the NOTCH pathway are common in this disease. In particular, mutations in the gene encoding NOTCH1 are present in almost one half of patients (3). Upon ligand binding, NOTCH1 is sequentially cleaved by ADAM and γ-secretase proteases. The cleaved NOTCH intracellular domain (NICD) translocates to the nucleus, where it associates with recombinant signal binding protein (RBP-J), and members of the mastermind-like (MAML 1–4) family, to turn on a transcription module vital for T-cell development. NOTCH1 mutations in patients with T-ALL activate downstream signaling in the absence of ligand (HD domain mutations), or extend the half-life of NICD (PEST domain mutations), leading to constitutive upregulation of the pathway (4).

In rare cases, similar pathway-activating mutations have also been discovered in NOTCH3 (5). In addition, mutations in FBXW7, the ubiquitin ligase that binds and degrades NICD, are found in 10%–15% of T-ALL cases (6); they increase the stability of NICD, thus allowing the NOTCH signal to persist. Because of the high prevalence of NOTCH pathway–activating mutations in this disease, translational efforts have been focused on inhibiting this pathway.

Gamma-secretase inhibitors (GSI) block NOTCH translocation to the nucleus and all downstream signals emanating thereof. They have been shown to induce cell-cycle arrest and, in some cases, apoptosis, and have demonstrated efficacy in mouse...
xenograft models of T-ALL (7). However, so far, GSIs have had limited success in patients. An initial clinical trial undertaken in the context of T-ALL demonstrated severe on-target gastrointestinal toxicities (8–10). In addition, a number of T-ALL cell lines as well as primary T-ALL cells are resistant to γ-secretase inhibition, in some instances due to loss of the tumor suppressor PTEN (11–13). Currently, patients depend solely on chemotherapy regimens and the prognosis of patients with relapsed or resistant T-ALL remains bleak.

In search of alternatives to conventional chemotherapy, we conducted a high-throughput drug screen across hundreds of human cancer cell lines (14). The screen revealed that both, GSI-sensitive and resistant T-ALL cell lines were highly sensitive to treatment with the BH3-mimetic, ABT-263. This sensitivity was due, at least in part, to low levels of MCL-1, an antiapoptotic protein that is known to counter the activity of ABT-263 (15, 16). Interestingly, our work revealed that blocking the NOTCH pathway in GSI-resistant models results in derepression of mTORC1, increased MCL-1 expression, and mitigated responses to ABT-263. Importantly, MCL-1 levels can be further lowered by pharmaceutical inhibition of mTORC1, enhancing ABT-263 activity in vitro and in vivo.

Materials and Methods

Information about cell lines, reagents, and immunoblotting experiments are detailed under Supplementary Information.

Cell lines were obtained from commercial sources (ATCC, DSMZ, and equivalent) and stocks created. Authentication of cell lines was further performed using short tandem repeat. All lines were tested for Mycoplasma routinely and only Mycoplasma-free cells were used. Cells were not kept in culture for extended periods of time. Typically, from frozen stocks derived from the commercial repository, cells were not kept in culture for more than 2 months.

High-throughput drug screen

Data for the 888-cell line screen were obtained through our collaborative HTS efforts (genomics of drug sensitivity in cancer: http://www.cancerrxgene.org).

Cell viability assays

HTS screening validation and further studies were performed as follows: 180 μL of a 4 × 10^3 cells/mL cell suspension was used to seed a 96-well plate. The next day, drugs were added to the cells, to a final volume of 200 μL/well. All drugs were serially diluted over a 9-point, 256-fold concentration range. Seventy-two hours after drug treatment, 50 μL/well of CellTiter-Glo (Promega) solution was added and the plate was read on a SpectraMax M5 ( Molecular Devices) luminometer. For 7-day assays, we followed the same protocol as above, except, a suspension of 1 × 10^5 cells/mL was used to seed the wells.

FACS apoptosis

Cells were seeded at 0.5 × 10^5 cells/well in 6-well plates. The next day, triplicate wells were treated with respective drugs. After 48 hours of treatment, cells were harvested, washed with PBS, resuspended in Annexin-binding buffer (BD Biosciences). Cells were stained with propidium iodide (BD Biosciences) and Annexin V Cy5 (Biovision) and analyzed on a LSRII flow cytometer (BD Biosciences).

Xenograft mouse studies

A suspension of 1 × 10^7 MOLT4 cells was inoculated subcutaneously into the left flanks of 6- to 8-week-old female athymic nude mice. Tumors were monitored until they reached approximately 500–800 mm^3. At this time, mice were randomized to control and treatment groups (n = 4–5/group). All drugs were administered once a day by oral gavage. ABT-263 was dissolved in a mixture of 60% Phosal 50 PG, 30% PEG 400, and 10% EtOH and administered at 80 mg/kg. AZD8055 was dissolved in Captisol, and administered at 16 mg/kg. AZD8055 was administered 1.5 hours prior to ABT-263 for combination treatments. Tumors were measured twice weekly using calipers. For pharmacodynamic analyses, tumor-bearing mice were administered with drugs or vehicle for 3 days. Three hours after the last treatment, tumors were excised and snap-frozen in liquid nitrogen for immunoblotting. All experiments were approved by the Massachusetts General Hospital Animal Care and Use Committee.

In vivo studies with primary T-ALL patient-derived xenografts

NOD-scid Il2rg-/- (NSG) mice were intravenously injected with 1 × 10^6 primary human T-ALL blasts. Once the leukemic burden reached 55% (TALL-X-7) or 65% (TALL-X-2) human leukemic blasts (as determined by hCD45 staining) in the peripheral blood, mice were randomized to one of four treatment groups. Vehicle (Captisol and 60% Phosal 50 PG, 30% PEG 400 and 10% EtOH), AZD8055 (16 mg/kg, diluted in Captisol), ABT-263 (80 mg/kg, diluted in 60% Phosal 50 PG, 30% PEG 400, and 10% EtOH) or both AZD8055 and ABT-263 were administered by oral gavage for 3 weeks using a 6-day on, 1-day off regimen. Mice were monitored daily and sacrificed when moribund. To assess leukemic burden, animals were sacrificed following 2 weeks of treatment and the percentage of human CD45+ leukemic cells in mouse spleen, bone marrow, and peripheral blood were determined by flow cytometry. All mouse procedures used in these PDX studies are approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. Primary T-ALL sample collection and in vitro studies are described under Supplementary Information.

Results

T-ALL cell lines are sensitive to ABT-263

Data from a high-throughput screen that we performed (14), wherein we tested the efficacy of 130 drugs on a panel of 888 human cancer cell lines, uncovered that T-ALL lines are highly sensitive to the BH-3 mimetic, ABT-263 (mean IC_{50} = 0.1 μmol/L for T-ALL lines, 0.8 μmol/L for other blood cell
Figure 1.
GSI-resistant cell lines are sensitive to ABT-263. **A**, Sensitivity values (log₁₀ IC₅₀ μmol/L) of 888 human cell lines treated with ABT-263, comparing T-ALLs, other leukemic and lymphatic lines (blood), and solid tumors. Each dot represents one cell line and each red bar is the geometric mean for that group. An unpaired \( t \) test was used to assess the differences in mean IC₅₀ between the T-ALLs and the other two groups. Data obtained from our high-throughput screen collaboration—Genomics of Drug Sensitivity in Cancer (https://www.cancerrxgene.org/). (Continued on the following page.)
Low MCL-1 levels are responsible for hypersensitivity to ABT-263

ABT-263 targets several antiapoptotic BCL2 family members such as BCL-2, BCL-XL, and BCL-w, but not others such as MCL-1 and BCL2A1 and high expression of MCL-1 is known to confer resistance to ABT-263 (15, 16, 23, 24). Previous data have shown that upon inhibition of BCL-2/BCL-XL/BCL-w with ABT-263, MCL-1 can sequester the newly released BIM from BCL-2/BIM and BCL-XL/BIM complexes, preventing free BIM-initiated apoptosis (25). Consistent with these findings, multivariate analysis of expression and mutational data of our large cell line collection identified MCL-1 as the top resistance genomic feature explaining response to ABT-263 in our high-throughput screen (14, 26). To investigate the relevance of BCL-2 family members in regulating response to ABT-263 in T-ALL, we analyzed the expression of BCL-2 family proteins (BCL-2, BCL-XL, MCL-1, and BIM) across a group of blood cancer cell lines including the 11 T-ALL models (Fig. 2A). Quantitation of the Western blots indicated that the levels of MCL-1 protein were considerably lower in the T-ALL cell lines examined compared with samples from other leukemic cell lines (Fig. 2B). In addition, analysis of our mRNA expression dataset across cell lines (http://www.cancerrxgene.org) revealed that levels of MCL-1 mRNA were significantly lower in the T-ALL lines compared with 145 lines from other blood cancers (Supplementary Fig. S3A). These results suggested that low MCL-1 levels underlie ABT-263 sensitivity in T-ALL. Intriguingly, while the GSI-sensitive lines are somewhat more sensitive to ABT-263 than the GSI-resistant ones, they have higher levels of MCL-1 than the GSI-resistant T-ALLs. We and others had previously shown that the ratio of BIM/MCL-1 is an important determinant of response to ABT-263 (27, 28). We thus examined levels of the proapoptotic protein BIM in these lines. The GSI-sensitive lines have higher levels of BIM, than the GSI-resistant lines, which could counterbalance for the higher levels of MCL-1 in these lines. Indeed, the BIM/MCL-1 ratio is significantly higher for the T-ALL lines as compared with the other leukemic lines (Fig. 2A and B). Furthermore, the BIM/MCL-1 ratios of the GSI-sensitive T-ALLs are similar to those of the GSI-resistant T-ALLs (Fig. 2A and B). Thus, differences in MCL-1 levels are often counterbalanced by differences in BIM levels and the BIM/MCL-1 ratio best explains ABT-263 sensitivity profile of the T-ALL lines.

To confirm that MCL-1 protein levels influence ABT-263 sensitivity in T-ALL, we cotreated the cell lines with ABT-263 and an MCL-1-specific inhibitor, A-1210477. Cells were further sensitized to ABT-263 in the presence of the MCL-1 inhibitor, with several cell lines showing high synergy (Fig. 2C; Supplementary Fig. S3B). It was not immediately obvious as to why cells with very low levels of MCL-1 were responding robustly to the combination of ABT-263 and the MCL-1 inhibitor. To gain insights into the

(Continued)
Figure 2.
Low MCL-1 levels are responsible for hypersensitivity to ABT-263. A, Fifteen micrograms of cell extract from each of the GSI-sensitive (green) and GSI-resistant (red) T-ALL lines and 7 other leukemic cell lines (blue) was separated by SDS-PAGE and probed with the indicated antibodies. The cell lines in blue represent B-ALL (GR-ST, LC4-1, P30-OHK), CML (JURL-MK1, KU-812), and CLL (JVM-2, EHEB). Independent experiments were performed at least three times and a representative result is shown. B, Scatter plots representing quantitated amounts of MCL-1/BCL2/BCL-xL/BIM protein normalized to β-actin, for the 18 cell lines in A. The proteins were quantitated using GeneTools from SynGene. An unpaired t test was used to assess the significance of differences in mean between the two groups. C, T-ALL cell lines were treated for 72 hours with 3 μmol/L of the MCL-1 inhibitor A-1210477 (black), 30 nmol/L ABT-263 (red), or a combination of 3 μmol/L of A-1210477 and 30 nmol/L ABT-263 (gray). Viability was measured using CellTiter-Glo and normalized to DMSO-treated samples. D, Two human T-ALL cell lines, MOLT-4 and RPMI-8402, were infected with lentiviral particles expressing GFP alone (GFP) or GFP-IRES-MCL-1 (MCL-1). GFP-expressing cells were sorted and tested for sensitivity to ABT-263 alone or in combination with A-1210477 (the MCL-1 inhibitor). Dose-response curves of the MCL-1-overexpressing lines treated alone (green) or in combination (gray) and the controls (orange) are shown. The experiments were done two times and the values shown are mean ± SD. Equal amounts of lysates from these lines were separated by SDS-PAGE and probed with MCL-1 and α-tubulin antibodies.
mechanisms leading to synergy, we treated the T-ALL lines with ABT-263 and examined MCL-1. As noted previously (25, 27, 29), MCL-1 levels increase in almost all the lines treated with ABT-263 (Supplementary Fig. S4). The MCL-1 inhibitor by itself was not effective in these lines (Fig. 2C). On the other hand, low-dose ABT-263 was potentiated by MCL1 inhibition. The effect of MCL-1 inhibition is thus likely through compensation of the MCL-1 increase induced by ABT-263. In fact, in cell lines such as PF-382 and CCRF-CEM, which show little increases in MCL-1 levels, the combination is not very effective (57% and 80% viability, respectively) and does not provide benefit over single agent ABT-263.

Furthermore, we overexpressed MCL-1 in two cell lines and examined sensitivity to ABT-263. Cell lines overexpressing MCL-1 were more resistant to ABT-263 than the parental and control lines overexpressing GFP (Fig. 3D). The MCL-1-overexpressing lines were resensitized to ABT-263 in the presence of the MCL-1 inhibitor. These results show that MCL-1 expression levels are a major determinant of the sensitivity of T-ALL cells to ABT-263. In addition, targeting MCL-1 can further sensitize T-ALL cells to ABT-263.

**NOTCH inhibition desensitizes T-ALL to ABT-263**

Because GSIs are used to limit the growth of T-ALLs, we combined ABT-263 with GSIs to see whether there was a further effect on viability. We treated human T-ALL cell lines with a well-studied GSI, compound E (CompE), for 3 days before exposing the cells to ABT-263. Interestingly, rather than sensitizing the cells, GSI treatment rendered the GSI-resistant lines 10- to 100-fold more resistant to ABT-263 (Fig. 3A and C). Furthermore, while in the case of GSI-sensitive lines the effect on ABT-263 was limited, the effects of ABT-263 by using drug combinations. Indeed, treatment with the mTOR inhibitor, AZD8055, which decreased phosphorylation of 4EBP1 and S6, also lowered MCL-1 expression in T-ALL cell lines (Supplementary Fig. S6B). To determine whether the regulation of MCL-1 protein levels by the NOTCH pathway (Fig. 3D) is through mTORC1, we treated GSI-resistant cell lines with CompE and measured mTORC1 activity. Indeed, treatment with a GSI led to decreases in intracellular NOTCH1, and concomitant increase in mTORC1 activity, as measured by phosphorylation of 4EBP1 and ribosomal protein S6 (Fig. 4A). The cell line that showed larger changes in MCL-1 levels (RPMI-8402; Fig. 4A and B), also exhibited the highest increase in phospho-4EBP1. To confirm that NOTCH1 does indeed suppress mTORC1, we knocked down NOTCH1 in one of the cell lines and examined whether we recapitulate the results seen with GSI inhibitors. Indeed, knockdown of NOTCH1 led to increased mTORC1 activity and increased MCL-1 levels (Supplementary Fig. S6C) and was accompanied by loss in sensitivity to ABT-263 (Supplementary Fig. S6D and S6E).

These results were intriguing, as previous studies had shown that NOTCH1 activates the PI3K–AKT pathway, leading to mTORC1 activation (11, 13). Notably, those studies were performed in PTEN-competent cells with NOTCH1 acting via PTEN to regulate changes in AKT activity (13). In contrast, the cell lines of interest here are GSI-resistant lines that are functionally PTEN null (13). In agreement with previous results showing positive regulation of AKT by NOTCH1, when NOTCH1 was inhibited in GSI-sensitive and PTEN wild-type cell lines, we observed an upregulation of PTEN and suppression of mTORC1 and MCL-1 (Fig. 4C). It is unclear at this point why ABT-263 sensitivity is marginally lowered by GSI treatment in the GSI-sensitive cells. It is possible that after GSI treatment, these GSI-sensitive cells are less prone to apoptosis.

To gain insights into how the NOTCH pathway regulates mTORC1, we examined the levels and activation status of regulators of mTORC1. Interestingly, based on the phosphorylation of AKT itself and two of its known substrates, ATP-citrate lyase and GSK3α/β (Supplementary Fig. S6F), AKT activity was not consistently modified by GSI treatment across the models. In one cell line (PF-382), AKT was activated upon GSI treatment, while in others (MOLT-4 and RPMI-8402) it was inhibited, and in yet another cell line (CCRF-CEM), it appeared unchanged. Thus, AKT regulation did not seem to explain the consistent activation effect seen on mTORC1 in the presence of GSI. Furthermore, we did not see consistent changes in the phosphorylation of TSC2 or AMPK, major upstream regulators of mTORC1 (Supplementary Fig. S6F). Interestingly, a less well-characterized regulator of mTORC1, REDD1, was seen as the best candidate to explain changes in mTORC1 activity upon GSI treatment. REDD1 was previously shown, in other contexts, to be a negative regulator of mTORC1 and exert its effect on mTORC1 by activating TSC1/TSC2 (without change in TSC expression; ref. 34). Indeed, in T-ALL cell lines too, knocking down REDD1 led to increased mTORC1 activity as well as higher levels of MCL-1 (Fig. 4D). Furthermore, previous studies have shown that the NOTCH pathway regulates REDD1 (encoded by the DDIT4 gene; refs. 35, 36). In three of the four cell lines, we observed that REDD1 levels decreased upon GSI treatment (Fig. 4A). However, one cell line (CCRF) showed increased mTORC1 activity without changes in REDD1 levels, suggesting that other factors regulate mTORC1 activity in those cells. Interestingly, REDD1 levels were also lowered in two PTEN-competent
models (Fig. 4C). However, this did not translate into higher mTORC1 activity in those models. This might be due to the PTEN-AKT regulation of mTORC1 overcoming the effect of REDD1 in those models (see pAKT308 in Fig. 4C). Overall, our data suggest that NICD suppression by GSI treatment and mTORC1 activation is due, at least in part, to a decrease in REDD1 levels. Consistent with previous findings (31, 32), mTORC1 suppression is accompanied by lowering of MCL-1 protein levels. In the context of already low MCL-1 protein levels due to low MCL-1 transcript levels, suppression of mTORC1 renders T-ALL cells highly sensitive to ABT-263.

Combination of AZD8055 and ABT-263 triggers high apoptosis in T-ALL cell lines and primary T-ALL samples in vitro

On the basis of our results so far, including the effect of mTORC1 inhibition on MCL-1 (Supplementary Fig. S6B), we tested whether we could further sensitize T-ALL to ABT-263 using an mTORC1 inhibitor. Indeed, the combination treatment of ABT-263 with the mTORC1 inhibitor AZD8055 resulted in decreased cell viability in both GSI-sensitive and resistant T-ALL cell lines (Supplementary Fig. S7A). In several lines, the combination synergistically reduced viability (Supplementary Fig. S7B). Consistent with apoptosis playing a major role in the combination
effect, the combined treatment of ABT-263 and AZD8055 led to massive apoptosis (90%) in most T-ALL models tested (Fig. 5A; Supplementary Fig. S8).

We next examined primary T-ALL samples obtained from patients at the time of diagnosis or relapse (Supplementary Fig. S9A). As shown previously, of the 6 primary T-ALL samples, 3 were GSI-sensitive and 3 were GSI-resistant (Supplementary Fig. S9B) (37). We note here that three of the models (TALL-X-12, TALL-X-13, and TALL-X-15) had wild-type NOTCH1, with low but detectable NICD (Supplementary Fig. S9A and S9C), and, as with established cell lines, there was no correlation between NICD levels and sensitivity to GSI (Supplementary Fig. S9B and S9C).

We treated all six primary T-ALL samples with vehicle, ABT-263, AZD8055, or a combination of the two and measured viability after 3 days of treatment. In all but one case, the combination treatment inhibited leukemic growth better than either single agent alone (Supplementary Fig. S10A). Annexin V/PI staining revealed that the combined therapy induced more apoptosis than either drug alone (Fig. 5B; Supplementary Figs. S11 and S12).

Interestingly, one of the models (TALL-X-15) was impervious to ABT-263 either as single agent or in combination with AZD8055 when measuring apoptosis (Fig. 5B; more in Discussion).

On the basis of this panel of cell lines and primary cultures, the combination effect over single-agent ABT-263 appeared more pronounced in the GSI-resistant than in the GSI-sensitive models (Fig. 5A and B). Indeed, ABT-263 treatment alone was very effective at killing the GSI-sensitive models tested (over 90% in several models), and the combination treatment could thus not increase apoptosis much further. This translates into higher level of synergy in the GSI-resistant models as
compared with the GSI-sensitive ones (Fig. 5A and B; Supplementary Fig. S10A).

We also analyzed lysates from one GSI-sensitive (TALL-X-7) and one GSI-resistant (TALL-X-2) sample after 6 hours of drug treatment. As with established cell lines, ABT-263 treatment increases MCL-1 levels in the primary T-ALLs too (Supplementary Fig. S10B). Furthermore, mTOR inhibition yields decreased phospho-S6 and phospho-4EBP1, and MCL-1 levels. In the combined treatment, mTOR inhibition likely acts synergistically with ABT-263 by preventing a surge in MCL-1, in some cases (TALL-X-2) yielding MCL-1 levels even lower than in untreated conditions (Supplementary Fig. S10B).

**Efficacy of the combination AZD8055 and ABT-263 in subcutaneous xenograft and primary patient-derived models**

We first examined efficacy of the combination treatment using a conventional subcutaneous xenograft model of the MOLT-4 cell line. Consistent with the *in vitro* findings, the growth of MOLT-4 leukemic cells was significantly impaired *in vivo* by treatment with ABT-263. Importantly, the combination of ABT-263 and AZD8055 was more efficacious than single agents alone and, in fact, led to tumor regressions (Fig. 6A). Pharmacodynamic studies of the tumor extracts indicate that AZD8055 effectively reduced mTORC1 activity and lowered MCL-1 levels (Fig. 6B).

We then tested the efficacy of ABT-263 and AZD8055 dual therapy in models of primary human T-ALL. We engrafted two primary T-ALL samples, one GSI-sensitive (TALL-X-7) and one GSI-resistant (TALL-X-2), into NOD-SCID Il2rγ-/- (NSG) mice. When leukemic burden reached 50%–65% CD45-positive human leukemic blasts in the peripheral blood, the mice were randomized to one of 4 treatment groups and treated for 21 days. Two to 6 mice from each treatment group were sacrificed after 14 days and effects on leukemic burden were determined (Fig. 6C). Mice were weighed regularly to evaluate whether the combination treatment was deleterious to their health; all treatments were well tolerated (Supplementary Fig. S13). Our results indicate that in the GSI-resistant model (TALL-X-2), the combination treatment significantly reduced leukemia burden and prolonged survival more effectively than either single agent alone (Fig. 6D; Supplementary Fig. S14). Consistent with the trend of GSI-sensitive models *in vitro*, treatment with the single-agent ABT-263 proved highly efficacious for TALL-X-7 *in vivo* (Fig. 6E; Supplementary...
Figure 6.

The combination of AZD8055 and ABT-263 causes tumor regression in vivo: GSI-resistant MOLT-4 cells were grown as xenograft tumors in Nu/Nu mice (A). Mice were randomized into 4 treatment cohorts: control (no drug), 16 mg/kg AZD8055, 80 mg/kg ABT-263, or their combination. Waterfall plot showing percentage change in tumor volume (relative to initial volume) for individual tumors in tumor-bearing mice, treated for 21 days. B, For pharmacodynamic studies, tumor-bearing mice were treated as in A, for 3 days. On the third day, tumors were harvested 2 hours after drug treatment and snap frozen. Proteins were extracted and 15 µg protein from each sample was run on an SDS-PAGE and subjected to Western blotting with the indicated antibodies. C, Schematic of the in vivo experiment with primary T-ALL cells. Briefly, NOD-SCID IL2Rγnull (NSG) mice were intravenously injected with primary human T-ALL blasts. When human leukemic blasts reached 55%–65% mouse peripheral blood, mice were randomized to one of four treatment groups indicated. Two to 6 mice from each group were sacrificed after 2 weeks of treatment to assess leukemic burden (data shown in Supplementary Figs. S10 and S11). The remaining mice were treated for a total of 3 weeks, after which survival was monitored. D and E, Kaplan-Meier survival curves are shown for mice engrafted with TALL-X-2 (D) or TALL-X-7 (E) patient samples. The difference in overall survival between the treatment groups was assessed by log-rank test (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001).
activate the PI3K pathway in wild-type and (REDD1). In another study, NOTCH inhibition was shown to tentatively observe AKT activation, leading us to study other possible treatment, in the series of models we studied we did not consistently observe AKT activation, upon manipulation of the NOTCH pathway was very pronounced in others. REDD1 is a stress-induced protein that is upregulated in response to many stimuli and is regulated by a number of transcription factors, including HIF1α, p63, p53, and ATF4 (34, 47–49). Because REDD1 is regulated by so many inputs, it is possible that the effect of NOTCH inhibition is not readily observed in some of the cell lines over other inputs. It is also possible that NOTCH regulates mTORC1 through other effectors in addition to REDD1 in these models. Regardless, our data support that mTORC1 is inhibited by NOTCH in GSI-resistant T-ALL lines, with REDD1 playing an important role connecting NOTCH and mTORC1 in many of these lines.

Our studies identify BCL-2/BCL-xL targeting as a candidate therapeutic strategy for the treatment of T-ALLs. In fact, TALL-X-15 primary patient model is the only one that, in our hands, is not sensitive to ABT-263. This clone harbors a TP53 loss-of-function mutation (p.H168P) and TP53 mutations in T-ALL are associated with high risk of relapse and very poor survival (50). This model was derived from a patient who relapsed after multiple failed treatments and it might have acquired additional mutations or epigenetic characteristics making it impervious to ABT-263.

We show that ABT-263-based therapies are effective in GSI-sensitive as well as GSI-resistant models. This is especially significant as preclinical studies and initial clinical indications strongly suggest that a number of tumors with active NOTCH pathways might be impervious to NOTCH inhibition (11). Furthermore, leukemic cells with mutations in FBXW7 are not responsive to GSIs (17). The three cell lines and one primary T-ALL model (X-7; Supplementary Table S1) with FBXW7 mutations in our study, respond well to ABT-263 and the combination treatment. Our work demonstrates that ABT-263 alone, or in combination with mTOR inhibitors, should be explored as a potential targeted therapy for patients with T-ALL. There is some indication from our studies that GSI-resistant tumors might benefit more from the combination therapy, whereas GSI-sensitive tumors respond well to ABT-263 alone. One possible approach to clinical deployment would be to test leukemic cells from patients ex vivo for sensitivity to GSI, ABT-263, and ABT-263 plus mTOR inhibitor and depending on the outcome, proceed with appropriate therapeutic strategy.

Disclosure of Potential Conflicts of Interest
J.A. Engelman has ownership interests (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

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Fig. S13). Absolute numbers, as well as percentages, of human CD45+ cells were significantly reduced in the blood, bone marrow, and spleen upon ABT-263 treatment and there was very little additional benefit with the combination, likely because of profound response to single-agent ABT-263 (Supplementary Fig. S15). This was also reflected in the survival curves for this model; while ABT-263 treatment significantly improved the lifespan of these leukemic mice, there was no added advantage with combination therapy compared with monotherapy (Fig. 6E). Taken together, our results suggest that ABT-263 is a promising therapeutic option as a single agent in some T-ALL, and that the addition of mTOR inhibitors could further improve efficacy, in particular, in GSI-resistant T-ALLs.

Discussion
ABT-263 has shown promising results in phase I clinical trials of relapsed or refractory CLL (38, 39), but has only been examined to a limited extent in the context of T-ALL (40, 41). Our studies show that low MCL-1 levels make T-ALL highly susceptible to ABT-263, whereas they are unsuitable for ABT-199 treatment, probably due to high levels of BCL-xL. While low MCL-1 mRNA levels likely lead to low protein levels, our work clearly shows that in GSI-resistant models, NOTCH represses mTORC1 and suppresses MCL-1. This crosstalk between two important growth pathways adds an interesting layer of regulation, where active NOTCH signaling leads to a further decrease in MCL-1 protein levels in T-ALL, making them vulnerable to ABT-263 therapy.

While the activation of AKT/mTOR by NOTCH1 has been described previously in GSI-sensitive, PTEN wild-type T-ALL models (11, 13), the repression of mTORC1 by NOTCH1 in GSI-resistant human T-ALLs is less well studied. Inhibiting PTEN described previously in GSI-sensitive, wild-type T-ALL models is more profound in Jurkat, CCRF-CEM and MOLT3, all PTEN-depleted, GSI-resistant lines (42). While this is in general agreement with our results showing an increase in mTORC1 activity upon GSI treatment, in the series of models we studied we did not consistently observe AKT activation, leading us to study other possible effectors of mTOR activation that NOTCH was affecting (REDD1). In another study, NOTCH inhibition was shown to activate the PI3K pathway in wild-type and KRASG12D murine models of T-ALL (43). It is somewhat surprising that two seemingly contradictory cross-talk events exist between NOTCH and the PI3K/mTOR pathway. On one hand, NOTCH activates AKT via PTEN suppression in some lines; on the other hand, NOTCH represses mTORC1 downstream of AKT and at least in part through REDD1. Notably, in addition to activating AKT/mTORC1, the product of PI3K, PIP3, plays many other roles (44–46). This network wiring might allow decoupling in part the PIP3 production from downstream mTORC1 activation.

Our work, as well as other reports, shows that the NOTCH pathway regulates REDD1 levels (35, 36). In our studies, the effect on REDD1 upon manipulation of the NOTCH pathway was very consistent and substantial across experiments in some models but less pronounced in others. REDD1 is a stress-induced protein that is upregulated in response to many stimuli and is regulated by a number of transcription factors, including HIF1α, p63, p53, and ATF4 (34, 47–49). Because REDD1 is regulated by so many inputs, it is possible that the effect of NOTCH inhibition is not readily observed in some of the cell lines over other inputs. It is
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