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Molecular Cancer Research

Perspective

Apoptotic Role of IKK in T-ALL Therapeutic Response

Irene Riz[1], Lynnsey A. Zweier-Renn[1,3], Ian Toma[2], Teresa S. Hawley[4], and Robert G. Hawley[1,3]

Abstract

Despite considerable progress in the treatment of T cell acute lymphoblastic leukemia (T-ALL), it is still the highest risk malignancy among ALL. The outcome of relapsed patients remains dismal. The pro-survival role of NOTCH1 and NFκB in T-ALL is well documented; also, both factors were reported to be predictive of relapse. The NOTCH1 signaling pathway, commonly activated in T-ALL, was shown to enhance the transcriptional function of NFκB via several mechanisms. Thus, pharmacological inhibition of NOTCH1-NFκB signaling was suggested to be incorporated into existing T-ALL treatment protocols. However, conventional chemotherapy is based on activation of various types of stress, such as DNA damage, mitotic perturbations or endoplasmic reticulum overload. NFκB is frequently activated in response to stress and, depending on yet unknown mechanisms, it either protects cells from the drug action or mediates apoptosis. Here, we report that T-ALL cells respond to NFκB inhibition in opposite ways depending on whether they were treated with a stress-inducing chemotherapeutic agent or not. Moreover, we found that NOTCH1 enhances NFκB apoptotic function in the stressed cells. The data argue for further studies of NFκB status in T-ALL patients on different treatment protocols and the impact of activating NOTCH1 mutations on treatment response. *Mol Cancer Res; 9(8); 979–84.* ©*2011 AACR.*

The NFκB transcription factor family is associated with a broad spectrum of human cancers and inflammatory diseases. It was shown to be required for viability of leukemic cells and recently suggested as a predictor of relapse in T cell acute lymphoblastic leukemia (T-ALL; refs. 1–3). The NOTCH1 signaling pathway, which is commonly activated in T-ALL (4), was demonstrated to enhance transcriptional function of NFκB via several mechanisms, including transcriptional activation and nuclear retention of the NFκB subunits, activation of upstream regulators, and a mechanism that prevents termination of NFκB signaling (1, 2, 5).

Combination therapy is the standard approach used to treat T-ALL (6). Thus, it has been suggested that inhibition of both NOTCH1 and NFκB should be used in combination with existing therapeutic agents (1, 2). However, the mechanism of action of ionizing radiation and the majority of conventional chemotherapeutic drugs are based on activation of various types of stress, such as DNA damage, mitotic perturbations, and the unfolded protein response. Frequently, NFκB is activated as a part of the stress response and, depending on yet unknown factors, it either protects cells from the drug or mediates apoptosis (7, 8). Here we report findings from a study in which T-ALL cells responded to NFκB inhibition in opposite ways depending on whether they were treated with a stress-inducing chemotherapeutic agent. Moreover, NOTCH1 enhanced NFκB proapoptotic function in the stressed cells. Our data are in agreement with clinical observations in which T-ALL patients who carry mutations that activate NOTCH1 exhibit a better initial response to treatment (9), suggesting that mutant NOTCH1 contributes to chemotherapy-induced cell death. Thus, we raise the question: Is it beneficial to inhibit the NOTCH-NFκB pathway in combination with conventional stress-inducing anti-T-ALL therapies?

As examples of stress-inducing therapeutic agents, we used etoposide, bortezomib, and vincristine. Etoposide is a topoisomerase II inhibitor that activates a stress response to DNA double-strand breaks and is a key component of reinduction chemotherapies that are being evaluated to treat relapsed T-ALL patients (10). Bortezomib, a proteasome inhibitor that activates the unfolded protein response, is also being evaluated clinically for relapsed ALL patients (ClinicalTrials.gov, Identifier NCT00873093). Vincristine is an inhibitor of chromosomal spindle formation that causes stress associated with mitotic perturbations and is a central component of most anti-T-ALL protocols (6). To inhibit NFκB, we used BMS-345541, a highly selective inhibitor of IκB kinase (IKK), the major upstream regulator of NFκB. BMS-345541 was previously reported to have anti-T-ALL

Authors' Affiliations: [1]Departments of Anatomy and Regenerative Biology and [2]Medicine, [3]Graduate Program in Biochemistry and Molecular Genetics, and [4]Flow Cytometry Core Facility, George Washington University, Washington, DC

Corresponding Authors: Irene Riz and Robert G. Hawley, Department of Anatomy and Regenerative Biology, George Washington University, 2300 I Street NW, Washington, DC 20037. Phone: 202-994-3511; Fax: 202-994-8885; E-mail: anaixr@gwumc.edu or rghawley@gwu.edu

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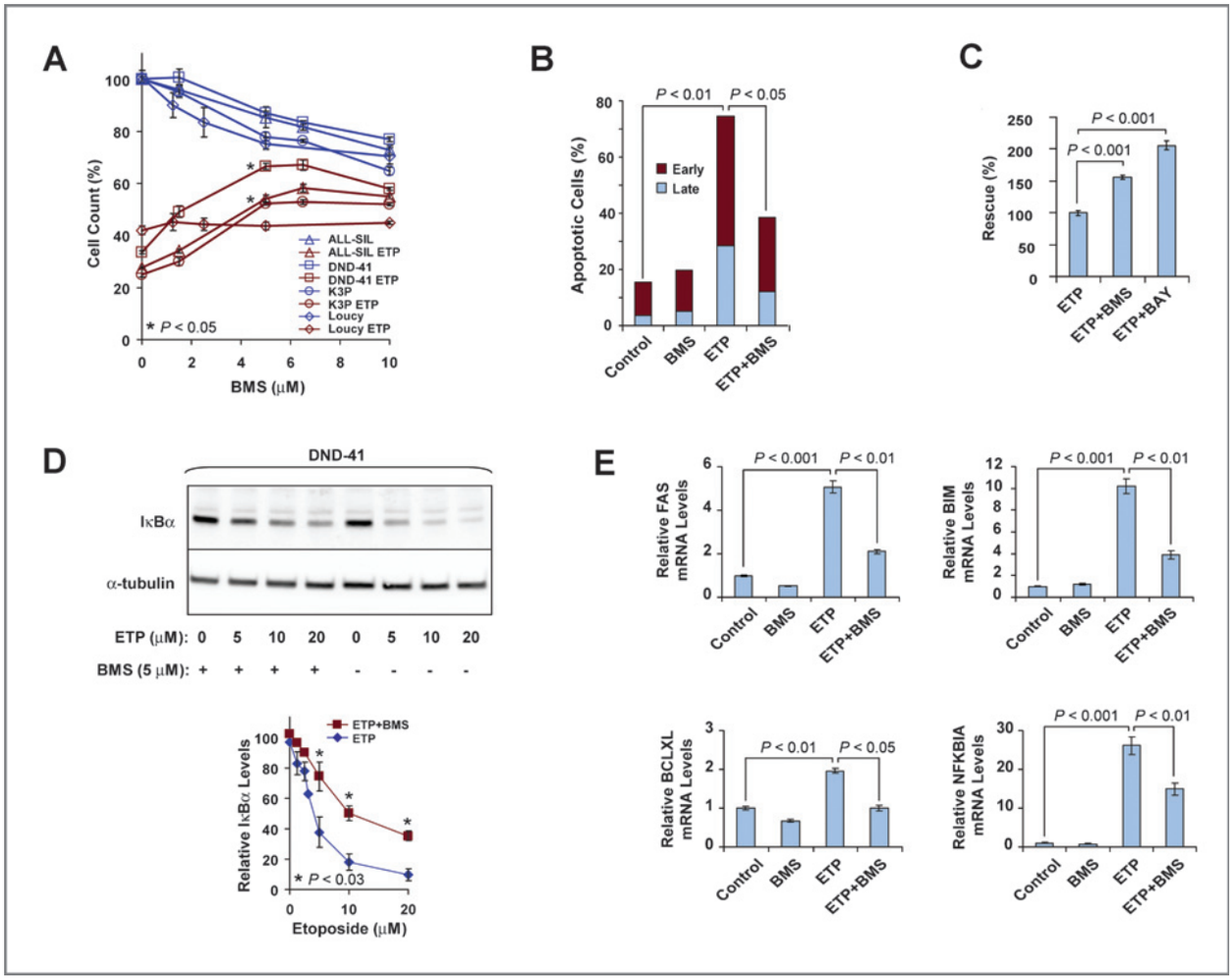

Figure 1. BMS counteracts ETP effects on cell growth, IKK activation levels, and NFκB target expression. A, alamarBlue cell growth assay (25). ALL-SIL, DND-41, K3P, and Loucy T-ALL cells, untreated or treated with 5 μM ETP and BMS at the indicated concentrations for 18 hours. *P* values indicate significant differences in cell counts for ETP-treated cells in the presence of 5 μM BMS (asterisks) versus ETP alone. Measurements were performed in triplicate. Similar results were obtained for DND-41 cells ($n = 5$) and ALL-SIL cells ($n = 2$) with different times of incubation, orders of ETP and BMS addition, and sources of serum. B, apoptosis assay (25). ALL-SIL cells were seeded at 2.5×10^5/ml, and early and late apoptotic cells were measured by flow cytometry after staining with annexin-PE and 7-AAD. Conditions: control, 0.06% dimethyl sulfoxide; ETP, 10 μM ETP; BMS, 10 μM BMS; and ETP+BMS, 10 μM ETP plus 10 μM BMS. Treatment was administered for 24 hours. A representative graph is shown for 2 biological replicates. Similar results were obtained for K3P cells. C, alamarBlue cell growth assay. DND-41 cells were treated with 5 μM ETP and with 8 μM BMS or 4 μM BAY 11-7082 (BAY) for 18 hours, as indicated. D, IKK kinase assay based on Western blot detection of IκBα degradation in DND-41 cells after 6 hours of treatment as indicated. *Left,* a representative blot of 3 independent experiments. *Right,* a quantitative analysis of IκBα levels demonstrating statistically significant partial inhibition of IκBα degradation for ETP-treated cells in the presence of 5 μM BMS (asterisks) versus ETP alone ($n = 3$). E. DND-41 cells were treated as described in D, and expression of the NFκB targets *FAS*, *BIM*, *BCLXL*, and *NFKBIA* was determined by qRT-PCR (19). Experiments were performed in triplicate for 2 biological replicates of 3 cell lines (ALL-SIL, K3P, and DND-41), and similar results were obtained. Student's *t*-test for all comparisons.

activity (1). We performed experiments with 5 cell lines derived from T-ALL patient samples: ALL-SIL, K3P, DND-41, HPB-ALL, and Loucy. We measured cell growth with alamarBlue, and apoptosis by annexin-V staining or detection of caspase-3 processing. We correlated the data with the expression levels of selected NFκB target genes and the levels of activation of the IKK complex (by detecting protein levels of the IKK substrate IκBα, which is degraded following phosphorylation) (7).

We found that partial inhibition of the IKK complex counteracted the cytotoxic effects of etoposide, bortezomib, and vincristine. Briefly, Fig. 1A shows growth response as a function of BMS-345541 concentration for 4 of the T-ALL–derived cell lines tested. We observed that BMS-345541 treatment alone caused inhibition of growth, consistent with the prosurvival role of NFκB in T-ALL reported by others (1, 2). However, when the cells were pretreated with etoposide, the response to BMS-345541 was altered: instead of

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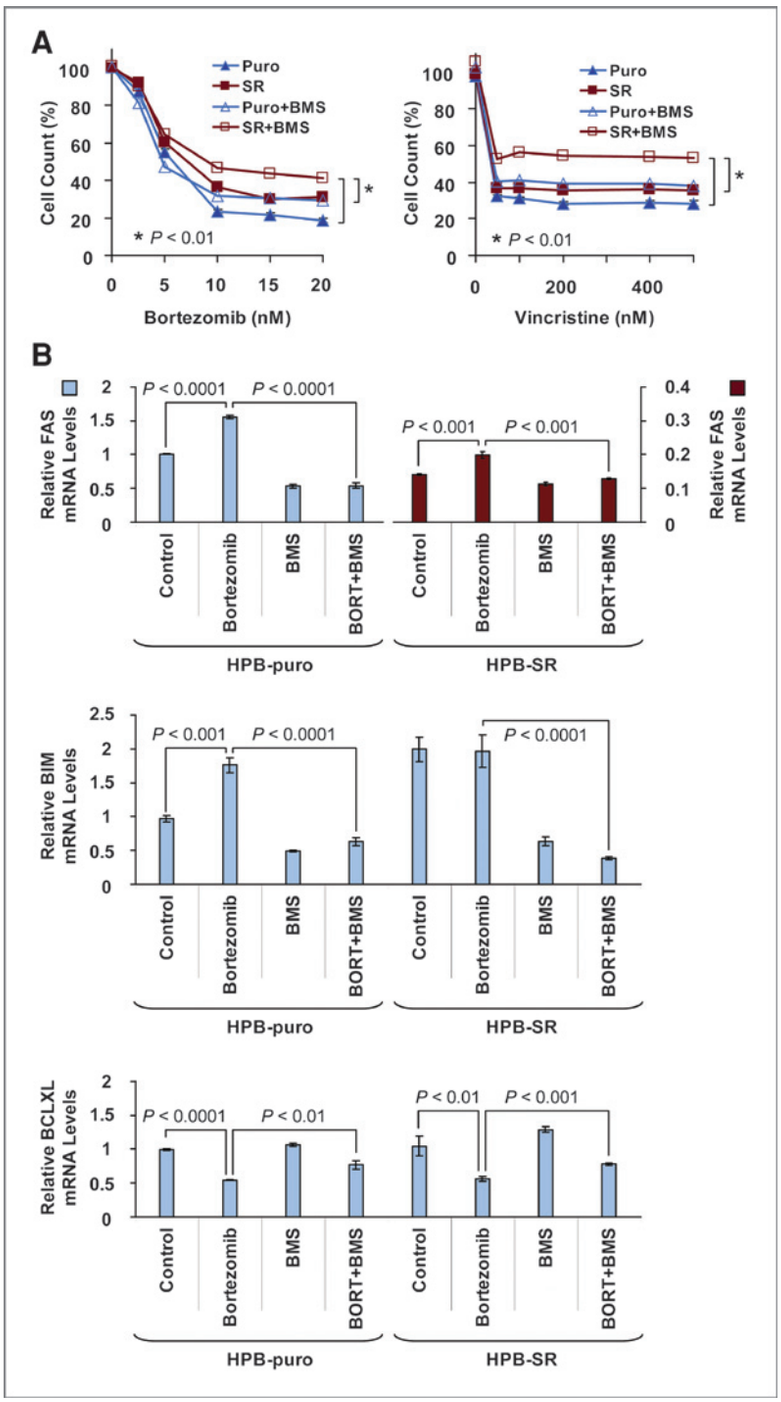

Figure 2. BMS counteracts bortezomib and vincristine effects on cell growth and NFκB target expression in HPB-ALL cells. HPB-SR cells (SR) express a *trans*-dominant superrepressor of IκBα (IκB-SR); HPB-puro (Puro) is an empty vector-transduced control. A, alamarBlue cell growth assay. Cells were treated with 5 μM BMS for 48 hours in the presence of bortezomib (*left*) or vincristine (*right*). *P* values indicate significant differences in cell counts at the highest concentrations of the drugs (asterisks). Experiments were performed in triplicate; representative graphs are shown. B. Expression of *BIM*, *FAS*, and *BCLXL* was determined by qRT-PCR (same conditions as in A). BORT, bortezomib. Experiments were performed in triplicate for 2 biological replicates. Student's *t*-test for all comparisons.

inhibiting growth, BMS-345541 treatment caused an increase in the number of ALL-SIL, K3P, and DND-41 cells, or (in the case of Loucy) had no effect (Fig. 1A). HPB-ALL cells were resistant to etoposide under the conditions tested (data not shown). We obtained essentially similar results when we measured apoptosis in ALL-SIL cells (Fig. 1B) and used 4 μmol/L BAY 11-7082 (another inhibitor of IκBα phosphorylation) in DND-41 cells (Fig. 1C). Etoposide was previously reported to activate NFκB (11). Consistent with this, we observed that etoposide activated the IKK complex (Fig. 1D), induced RELA nuclear translocation (not shown), and upregulated the NFκB target genes *FAS*, *BCL2L11* (*BIM*), *BCL2L1* (*BCLXL*), and *NFKBIA* (Fig. 1E). Treatment with 5 μmol/L BMS-345541 partially reversed

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these effects (Fig. 1D and E). In summary, our data indicate that etoposide-mediated IKK activation contributes to stress-induced T-ALL cell killing; thus, combining IKK inhibitors with etoposide may not be therapeutically beneficial.

Because HPB-ALL was resistant to etoposide under the conditions tested, we examined the effects of treating these cells with bortezomib and vincristine. As an additional approach to inhibit NFκB function, we expressed a *trans*-dominant superrepressor form of IκBα (IκB-SR) harboring 2 amino acid substitutions (S32A/S36A) that render it resistant to phosphorylation and degradation, and prevent a signal transfer from IKK to NFκB (12). Treatment with bortezomib and vincristine gave essentially similar results, consistent with activation of an IKK and NFκB proapoptotic function: BMS-345541 treatment and IκB-SR expression partially reversed the cytotoxic effects of both drugs (Fig. 2A). Of interest, the effects of BMS-345541 and IκB-SR expression were cooperative because HPB-ALL cells expressing IκB-SR that were also treated with BMS-345541 were the most resistant to bortezomib or vincristine (Fig. 2A). These findings suggest that the mechanism targeted by IKK inhibition may have an NFκB-independent component (7). Bortezomib treatment caused induction of the proapoptotic genes *FAS* and *BIM*, and repression of prosurvival *BCLXL*. These transcriptional responses were attenuated by BMS-345541 treatment and IκB-SR expression (Fig. 2B). In support of these data, bortezomib was recently shown to activate the IKK-NFκB pathway in other human malignancies (13). Although our observations indicate that etoposide action is mediated by IKK-NFκB transcriptional activation of apoptotic genes, other topoisomerase II poisons (such as doxorubicin) have been shown to induce IKK-NFκB–dependent apoptosis via repression of *BCLXL* (11), similar to what we observed for bortezomib. Despite differences in the downstream events, IKK activation contributed to apoptosis induced by all of the above therapeutic agents tested. Thus, inhibition of the IKK

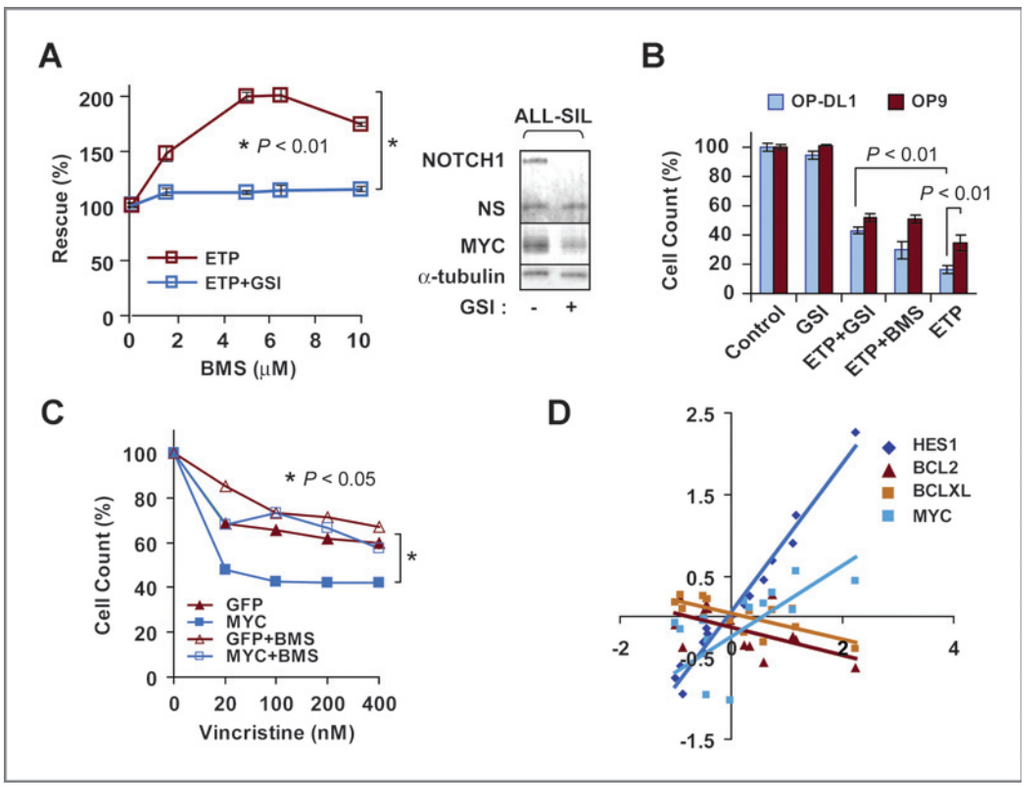

Figure 3. Role of the NOTCH1/MYC-NFκB pathway in T-ALL response to ETP and vincristine. NOTCH1 inhibition prevents the BMS rescue effect. A, *left.* After pretreatment of ALL-SIL cells in suspension cultures with 1 μM of the γ-secretase inhibitor (GSI) Compound E or 0.01% dimethyl sulfoxide for 10 days, cell counts were determined by alamarBlue assay. *P* value indicates significant difference in the presence of 5 μM BMS (asterisk). A, *right.* Western blot analysis shows inhibition of activated NOTCH1 and downregulation of MYC protein levels in GSI-treated ALL-SIL cells (19). NS, nonspecific band. Similar results were obtained for K3P and DND-41 cells. B, DND-41 cells were grown on monolayers for 3 days. The protective effect was more prominent in the case of cells cocultured with OP9-DL1 compared with OP9; 5 μM ETP, 5 μM BMS, 1 μM Compound E as indicated for 18-hour treatment. The experiment was performed in triplicate for 2 biological replicates. C, alamarBlue cell growth assay. Immortalized mouse hematopoietic progenitors overexpressing *MYC* (18, 19) were treated with vincristine in the presence or absence of 5 μM BMS, 18-hour treatment. GFP is an empty vector-transduced control. The experiment was performed in triplicate; a representative graph is shown. *P* value indicates significant differences in cell counts at the highest concentration of the drug (asterisk). Student's *t*-test for all comparisons. D. Expression of NOTCH1 target genes *HES1* and *MYC* inversely correlates with expression of *BCL2* and *BCLXL* prosurvival genes in T-ALL patient samples (23). Samples were ranked on *HES1* levels, and average values for groups of 6 were plotted. The correlation coefficients were determined for 2 independent HES1 probe sets as indicated in Table 1.

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Table 1. Expression of NOTCH1 target genes *HES1* and *MYC* inversely correlates with expression of *BCL2* and *BCLXL* prosurvival genes in T-ALL patient samples

HES1 affymetrix probes	Correlation coefficients			
	HES1	BCL2	BCLXL	MYC
203394_s_at	0.99	−0.56	−0.72	0.57
203395_s_at	1.00	−0.49	−0.68	0.61

pathway in T-ALL may not provide a therapeutic advantage when combined with these drugs.

To directly test the role of NOTCH1 in the NFκB proapoptotic response, we modulated NOTCH1 signaling by γ-secretase inhibition or by coculturing T-ALL cells on OP9-DL1 monolayers presenting the NOTCH1 ligand Delta-like 1 (14). Briefly, we treated ALL-SIL, K3P, and DND-41 cells with the γ-secretase inhibitor Compound E. After several days of treatment, we observed a decrease in etoposide-induced cytotoxicity concomitant with a reduced ability of BMS-345541 to counteract the cytotoxic effects (Fig. 3A). Thus, our data indicate that IKK's contribution to etoposide-induced T-ALL cell apoptosis is diminished by NOTCH1 inhibition. Conversely, we found that Delta-like 1–mediated activation of NOTCH signaling in OP9-DL1 cocultures enhanced the response to etoposide. BMS-345541 or Compound E treatment partially protected the T-ALL cells from etoposide-induced death under these conditions (Fig. 3B).

MYC is a downstream target of NOTCH1 in T-ALL (15, 16). Other studies have shown that enforced expression of *MYC* in hematopoietic cells enhances sensitivity to etoposide and other stress-inducing therapeutic agents (17). We observed that overexpression of *MYC* in immortalized mouse hematopoietic progenitors (18, 19) also increased sensitivity to vincristine (Fig. 3C). Moreover, the effect was alleviated by BMS-345541, suggesting that the MYC-enhanced response to vincristine is mediated by IKK (Fig. 3C). In this regard, the cytotoxic effects of vincristine on T-ALL cells were previously shown to be attenuated by γ-secretase inhibitor treatment (20). Although the mechanism remains to be defined, our data indicate that MYC is one of the downstream components of NOTCH signaling that contribute to the NFκB proapoptotic response to chemotherapy. Of note, activation of the IKK-NFκB pathway by vincristine was previously implicated in the induction of apoptotic cell death in other human tumor types; moreover, the investigators showed that glucocorticoids, which are known to partially act via inhibition of NFκB, attenuated vincristine-induced death (21). These findings might explain why additional courses of vincristine and dexamethasone combination therapy did not result in improved treatment outcomes in a recent worldwide survey of T-ALL clinical trials (22).

Therefore, our data suggest that NOTCH1 or MYC activation levels are potential candidates to predict the apoptotic role of NFκB in response to stress-inducing chemotherapy. We analyzed publicly available microarray profiling data from 92 T-ALL patients (23). We plotted the expression levels of *HES1* as a measure of NOTCH1 pathway activation versus prosurvival *BCL2* and *BCLXL* expression levels and found that they were inversely correlated throughout the range of NOTCH1 activation (Fig. 3D). As expected, *HES1* mRNA levels directly correlated with *MYC* mRNA levels, consistent with findings that NOTCH1 induces *MYC* expression in T-ALL cells (15, 16). These correlations support the notion that when NOTCH1 is activated, the balance of NFκB targets may shift toward an apoptotic outcome.

Despite an overall cure rate of 80% in children, the overall survival of adult ALL patients is only ∼35% (6). For both patient populations, relapse remains a serious problem in T-ALL. The fate of relapsed patients is dismal. Historically, to prevent relapse, T-ALL patients underwent an aggressive combination therapy that was so intense that toxicity became a significant cause of unfavorable outcome (24). New, more effective therapeutic agents and a better understanding of their compatibility with existing drugs are needed. We found that under the conditions examined in our study chemotherapy-induced stress changed the function of the NOTCH-NFκB signaling pathway in T-ALL cell lines from prosurvival to proapoptotic. Thus, the data argue strongly for further studies of the circumstances that determine NFκB action in T-ALL patients on different treatment protocols, and the impact of activating *NOTCH1* mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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