Ruxolitinib Synergizes With Dexamethasone for the Treatment of T-cell Acute Lymphoblastic Leukemia

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Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer and comprises B cell ALL (B-ALL, 85% of cases) and T cell ALL (T-ALL, 15% of cases).[1,2] Over the past years, optimization of multi-agent chemotherapy, improved supportive care, and risk group stratification have improved 5-year overall survival rates to more than 90% in children[3,4] and 50% in adults.[5,6] Nevertheless, relapse and failure to achieve clinical remission remain major challenges. Moreover, current treatment regimens are associated with severe short-term and long-term side effects, including life-threatening infections, osteonecrosis, neurobehavioral side effects and growth defects.[7] Targeted therapy has emerged as an alternative, less toxic treatment option by selectively targeting cancer cells with a specific mutation, and has been successfully introduced for the treatment of BCR-ABL1 positive B-ALL.[2,8]

Recent sequencing efforts have identified a variety of mutations that cause activation of the IL7R/JAK/STAT signaling pathway in ALL, which can potentially be targeted by JAK kinase inhibitors.[9,10] Mutations in the IL7R signaling pathway are associated with reduced steroid sensitivity and poor clinical outcome.[11] Pre-clinical studies suggest that ALL cases with alterations in JAK1, JAK2, JAK3, IL7R, DNM2, or CRLF2 can

be sensitive to existing JAK inhibitors.[12–14] Moreover, ETP-ALL cases were found to be sensitive to ruxolitinib independent of the presence of JAK/STAT pathway mutations.[15] In this study, we used the JAK1/JAK2 kinase inhibitor ruxolitinib in combination with dexamethasone, to treat the IL7R mutant ALL cell line DND-41 and JAK3 mutant patient derived xenograft samples (PDX). As JAK3 mutants are dependent on JAK1 signaling for their cellular transformation, it is possible to use both JAK1/JAK2 and JAK3-selective inhibitors on JAK3 mutation–positive leukemias.[16,17] Ruxolitinib is already approved for the treatment of MPN,[18] and is currently being evaluated for the treatment of B-ALL (NCT02723994).[19]

To identify efficient combinations of ruxolitinib with currently used chemotherapy, we tested for synergistic effects between ruxolitinib and dexamethasone, vincristine or doxorubicin. For our initial experiments, we used the IL7R mutant T-ALL cell line DND-41, which is sensitive to each of the drugs alone. DND-41 cells were treated with the single drugs or drug combinations for 48 hours and proliferation was measured using the ATPlite Luminescence Assay (PerkinElmer). Addition of ruxolitinib to dexamethasone resulted in a significant, dose dependent decrease in proliferation compared to dexamethasone treatment alone (Fig. 1A). When combining ruxolitinib with doxorubicin the synergistic effect on proliferation was less evident, with only the highest dose of 800 nM leading to decreased proliferation compared to the doxorubicin alone (Fig. 1B). Combining ruxolitinib with vincristine had no additional effect on DND-41 compared to vincristine alone (Fig. 1C).

We explored whether the observed effect on proliferation was also associated with increased apoptosis. DND-41 cells were treated for 48 hours with single compounds and the combinations of ruxolitinib with either dexamethasone, doxorubicin or vincristine. Flow cytometry analysis was performed on the cells for Annexin V and Propidium Iodide (PI). Treatment with dexamethasone alone slightly increased the percentage of apoptotic cells (Fig. 1D), and the combination of ruxolitinib with dexamethasone increased the percentage of apoptotic cells by 3-fold compared to single drug treatment (Fig. 1D). In contrast, combining ruxolitinib with doxorubicin had no synergistic effect on apoptosis (Fig. 1E) and the combination of ruxolitinib with vincristine was even antagonistic (Fig. 1F).

These data suggested synergy between dexamethasone and ruxolitinib, which was confirmed based on calculations of the combination index (CI). We used the Chou-Thalalay method
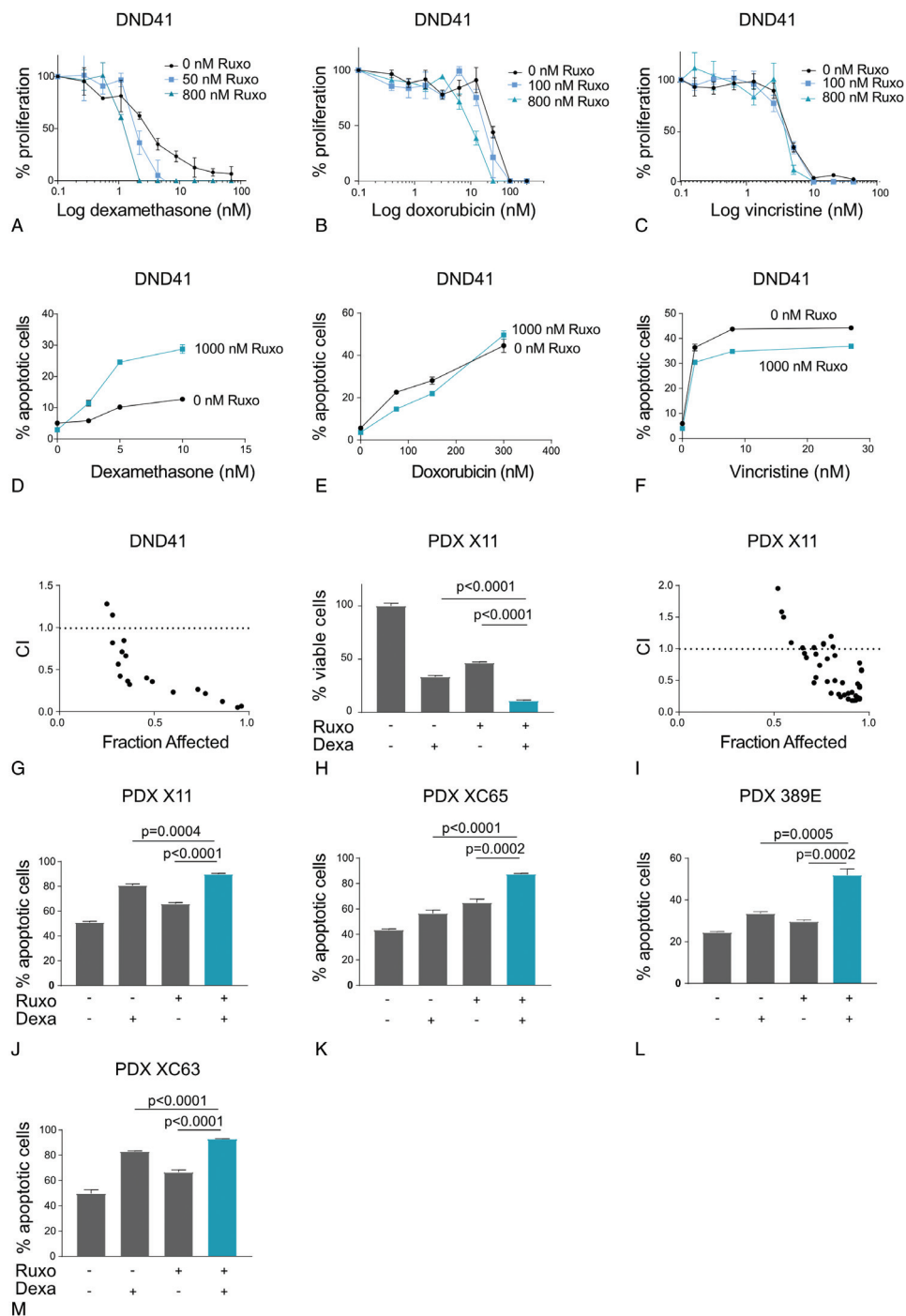
Figure 1. Effects of ruxolitinib combined with chemotherapy drugs on proliferation and apoptosis of in vitro cultured cells. (A) Proliferation analysis after ruxolitinib and dexamethasone combination treatment. The DND-41 cell line was treated with a dilution series of dexamethasone together with 0 nM, 50 nM, or 800 nM of ruxolitinib (Ruxo). DMSO was used as vehicle. (B) Proliferation analysis after ruxolitinib and doxorubicin combination treatment. The DND-41 cell line was treated with a dilution series of doxorubicin together with 0 nM, 100 nM or 800 nM of ruxolitinib (Ruxo). (C) Proliferation analysis after ruxolitinib and vincristine combination treatment. The DND-41 cell line was treated with a dilution series of vincristine together with 0 nM, 100 nM or 800 nM of ruxolitinib (Ruxo). (D–F) DND-41 cells were treated with increasing concentrations of dexamethasone (0-2-5-10 nM), doxorubicin (0-75-150-300 nM) or vincristine (0-2-8-27 nM), each time in combination with DMSO (vehicle) or Ruxolitinib (1000 nM). Apoptotic cell death was determined after 48 hours with annexin V-PI staining. Apoptotic cells were defined as annexin V+/PI- and annexin V+/PI+ cells. (G) Fraction affected - Combination index (CI) plot for synergy assessment. Cells were treated with a dilution series of dexamethasone and ruxolitinib for 48 hours, followed by proliferation measurement with ATP-lite. The different combinations were assessed using the Chou-Thalalay method and Compusyn software. A CI value below 1 indicates synergy. Very strong synergistic combinations have a CI value below 0.2. Antagonism is defined by a CI>1 and CI = 1 when the effect is additive. (H) Viability assessment of ex vivo treated patient sample X11 (JAK3 M511I). Ex vivo treatment was performed on single cells for 24 hours with 10 nM dexamethasone (Dexa) and 250 nM ruxolitinib (Ruxo) or a combination of both. The ATP-lite assay was used to determine viable cells. (I) Fraction affected - Combination index (CI) plot for synergy assessment of PDX X11 after 24 hours treatment with a dilution series of dexamethasone and ruxolitinib (J–M) Annexin V-PI staining after 24 hours of treatment ex vivo of the PDX samples X11 (JAK3 M511I), XC65 (JAK1(R724H) JAK3 (A573 V)), 389E (*JAK3*(M511I)), and XC63 (JAK3 (M511I)). Concentrations of ruxolitinib (Ruxo) and dexamethasone (Dexa) were respectively 500 nM and 80 nM for X11; 500 nM and 5 nM for XC65; 1000 nM and 10 nM for 389E; 500 nM and 20 nM for XC63. All experiments were performed in triplicate, p values were calculated with graphpad Prism using the Student *t* test.

and CompuSyn software,[20] which indicated CI values well below one, confirming that dexamethasone and ruxolitinib decrease proliferation in a highly synergistic way (Fig. 1G). Overall, we conclude that combination treatment in the DND-41 cell line was synergistic when ruxolitinib was added to dexamethasone, but not when ruxolitinib was added to vincristine or doxorubicin, where we observed the lack of effect or even antagonism.

Next, we tested if the synergy between dexamethasone and ruxolitinib was also observed during treatment of JAK3 mutant patient-derived T-ALL xenograft (PDX) samples ex vivo. We selected PDX samples with different JAK3 mutations (PDX-X11: JAK3(M511I), PTPRC(R680C), SETD2(G93S), WT1(fs aa369), CTCF(splice aa453), EP300(M126V), PHF6(H302-Y303insERFG*), deletion CDKN2B; PDX-389E: JAK3 (M511I), DNM2(splice), NOTCH1(L1600P); PDX-XC65: JAK1(R724H), JAK3(A573V), NOTCH1(fs aa2438); PDX-XC63: JAK3(M511I), NOTCH1(L1678P), NOTCH1 (Q2459*)). All PDX and in vivo experiments were approved by the ethical committee of the University of Leuven. Human leukemic mononuclear cells were injected through tail vein injection into 6 to 12 week old Non-obese diabetic.Cg-prkdc^{scid}il2rg^{tm1wjl}/szj (NSG) mice. Expansion of the human leukemic cells was monitored by staining peripheral blood samples with human anti-CD45 (hCD45) antibody. Once hCD45 levels in the blood reached 50 percent, the human leukemic cells were collected from the spleen. The fresh single cells were resuspended in RPMI1640 with 20% fetal bovine serum and treated for 24 hours with dexamethasone, ruxolitinib or a combination of both in 5% CO_2 at 37°C.

Treatment of the PDX-X11 cells for 24 hours with dexamethasone monotherapy induced a stronger reduction of cell viability than ruxolitinib, and the combination of dexamethasone with ruxolitinib was again more potent to reduce viability compared to the single agents (Fig. 1H). We next calculated the CI values and confirmed ex vivo that the decreased viability was again due to synergistic interaction between dexamethasone and ruxolitinib (Fig. 1I). This is in agreement with a study on genetically engineered IL7R and JAK1 mutant cell lines that showed increased steroid sensitivity when combined with ruxolitinib.[11] We measured if this decreased cell viability was due to apoptosis by performing Annexin V and PI staining after 24 hours of treatment. Similar to our results from the DND-41 cell line, we observed a mild but significant increase in apoptosis when combination therapy was used compared to monotherapy (Fig. 1J). We tested if this synergy was also observed with other PDX samples by using apoptosis as a readout. All three additional PDX samples (XC65, 389E, XC63) underwent more apoptosis after combination treatment compared to single treatment (Fig. 1K–M). Our different PDX samples showed that each sample with its own distinct mutational profile responds differently to the single and combination therapy, but that the combination therapy consistently caused more apoptosis across all samples.

We validated these findings also in vivo using 2 T-ALL PDX models. For this, we first injected NSG mice with the PDX-X11 sample, in which we introduced GFP/luciferase expression via lentiviral transduction. Engraftment was assessed via bioluminescent imaging (BLI). After 18 days when engraftment was clearly detected (determined as total flux (photon/sec) per mouse >10^7), mice were randomized in four groups with equal distribution of BLI and weight and treatment was started. Mice were treated for 2 weeks with vehicle, dexamethasone, ruxolitinib or the combination of ruxolitinib with dexamethasone. As we noticed toxicity

with continuous treatment, dexamethasone was given at a concentration of 4 mg/L in the drinking water for 3 days, followed by two days of water without dexamethasone. Previous research had shown that discontinuous and continuous treatment reached equal efficacy.[21] Ruxolitinib was given once a day for 14 consecutive days at a dose of 50 mg/kg. BLI was performed before the start of the treatment, after 5 days of treatment and at the end of treatment (day 12). After 14 days, mice were sacrificed and organ infiltration was assessed (Fig. 2A).

BLI showed an increase in the leukemic burden over time (Fig. 2B, C). After 5 days of treatment, ruxolitinib monotherapy had less effect compared to dexamethasone monotherapy, with both treatments leading to leukemia expansion. In contrast, mice treated for 5 days or 12 days with the combination of ruxolitinib and dexamethasone showed less leukemic progression compared to single drug treatment (Fig. 2B-C). After 14 days of treatment, we euthanized the animals and analyzed leukemia cell infiltration in various organs. There was almost no reduction of leukemic cells in the peripheral blood with ruxolitinib monotherapy, while dexamethasone treatment had reduced the percentage of human leukemia cells significantly compared to placebo treated mice (Fig. 2D). Combination therapy further reduced the leukemic cells in the blood, although not significantly compared to dexamethasone (Fig. 2D). Spleen weight was significantly reduced with ruxolitinib alone. Dexamethasone and the combination treatment both reduced spleen weight even further to normal levels (Fig. 2E). Despite the suppression of splenomegaly, there was still leukemic infiltration in the spleen (Fig. 2F), with the lowest levels measured for the animals treated with combination therapy. Importantly, the combination treatment also showed the strongest reduction of leukemia cells in the bone marrow (Fig. 2G). Ruxolitinib alone could only weakly reduce leukemia cells in the bone marrow (20% reduction) compared to placebo treated mice, while dexamethasone treatment showed stronger effects (50% reduction). The combination of dexamethasone with ruxolitinib could reduce the leukemia cells in the bone marrow with more than 80%, which was significantly better than each of the other regimens (Fig. 2G). A second in vivo mouse model PDX 389E, was treated for 3 weeks following the same treatment scheme as for PDX X11. For PDX 389E all treatments had a mild effect on peripheral blood counts and we did not measure a significant reduction of leukemic blasts in the blood (Fig. 2H). Despite the overall mild effects, there was a clear benefit of the combination treatment on spleen weight. Dexamethasone or ruxolitinib alone caused a reduction of spleen weight and the combined treatment led to a further significant reduction (Fig. 2I).

Delgrado-Martin et al previously described via in vitro PDX models that it would be beneficial to add ruxolitinib to dexamethasone. In their study, they focused on IL7-dependent dexamethasone resistance.[22] We did not assess the IL7 responsiveness in our patient samples, but instead we focused on samples with mutations in the IL7R-JAK-STAT pathway to determine synergy in the DND-41 cell line in vitro and in T-ALL PDX samples both in vitro and in vivo. All our experiments were independent of the presence of human IL7. The mutational status of the patients is more likely to be tested in the clinic compared to testing the IL7 dependency. The ETP status and IL7 dependency could be exploited as additional markers for patients who are negative for IL7R-JAK-STAT mutations.[15,22] Complementary to the previous observations that IL7 was not able to protect PDX samples from death induced by vincristine,[22] we did not observe synergy between ruxolitinib and vincristine nor doxorubicin in
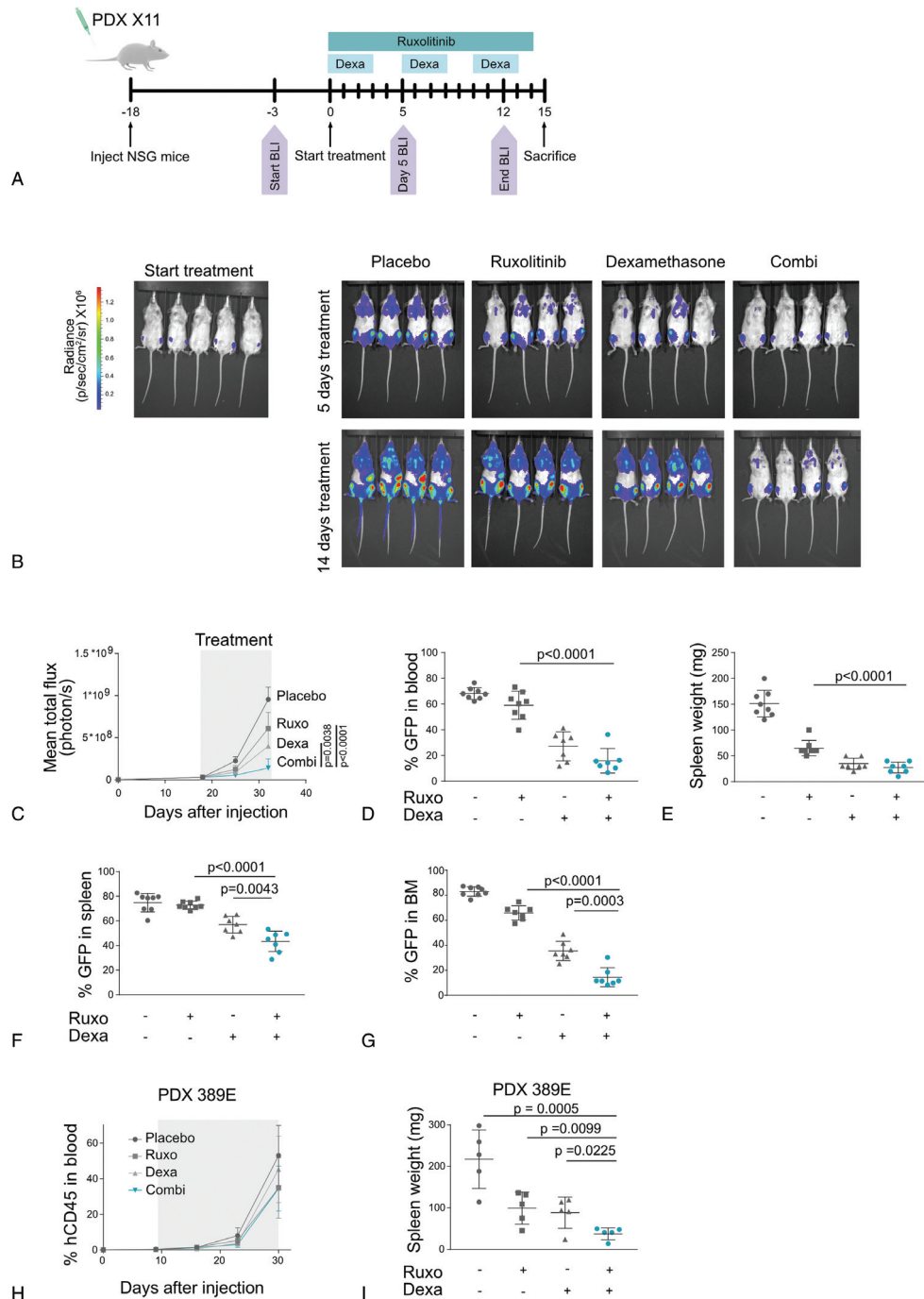
Figure 2. In vivo treatment of a patient-derived T-ALL xenograft with ruxolitinib combined with dexamethasone. (A) Timeline of PDX X11 treatment. 10^6 luciferase and GFP positive PDX X11 cells were injected in the tail vain of 6 to 12-weeks old NSG mice. After two weeks, we assessed disease burden by performing bioluminescent imaging (BLI). All mice had reached a total flux of $>10^7$ photon/sec. Treatment with dexamethasone (Dexa) and ruxolitinib (Ruxo) was started 18 days after injection. Ruxolitinib was given at a dose of 50 mg/kg for 14 consecutive days. Dexamethasone was given in the drinking water at a dose of 4 mg/L for 3 days, followed by 2 days without dexamethasone. Five and 12 days after start treatment BLI was performed and mice were sacrificed after two weeks of treatment to assess organ infiltration by leukemia cells. $N = 8$ for placebo and ruxolitinib and n = 7 for dexamethasone and combination. (B) Bioluminescent imaging of PDX X11 at different time points during treatment. Images were taken at the start of the treatment, after 5 days and at the end of treatment. The images shown are of representative mice. Mice were imaged with the IVIS Spectrum in vivo Imaging System (PerkinElmer). (C) Overview of the BLI flux evolution. Average of the mean total flux (photon/sec) over time is shown for the different treatment groups. (D) Percentage of GFP positive cells in the peripheral blood for each treatment group. Peripheral blood withdrawal was performed before sacrificing the mice. GFP percentage was measured via flow cytometry on a MACSQuant Vyb (Miltenyi). (E) Spleen weight (mg) after indicated treatments. (F) Single cells were made from total spleens after which we measured the percentage leukemic GFP positive cells in the spleen via flow cytometry. (G) Leukemic bone marrow cells were flushed from the bones and percentage GFP analyzed via flow cytometry. (H) Overview of leukemic infiltration in the blood after injection of 389E PDX in vivo in NSG mice. Average of the percentage hCD45 over time is shown for the different treatment groups. Grey box indicates the treatment period (n = 5). (I) Spleen weight (mg) of mice injected with 389E PDX after indicated treatments. p values were calculated with graphpad Prism using the Student *t* test.

our ALL models. This is also in line with studies on other kinase inhibitors where the combination with chemotherapy was sometimes even antagonistic.[23] Dexamethasone activates the glucocorticoid receptor, which activates several target genes that will result in less proliferation and more apoptosis. Ruxolitinib will have a similar effect through inhibition of the JAK/STAT pathway. Since dexamethasone and ruxolinib function through different pathways, we can expect synergy, which we indeed observed. A recent study also showed that a new anti-IL7R antibody sensitized T-ALL cells to dexamethasone treatment.[24]

In conclusion, we demonstrate synergy between ruxolitinib and dexamethasone in pre-clinical ALL models in vitro and in vivo. Our data support that combined treatment with ruxolitinib and dexamethasone leads to a stronger reduction of leukemia cell growth and enhanced apoptosis compared to single drug treatment. Our data indicate that ruxolintinib can enhance the anti-leukemia effect of dexamethasone, which could translate in stronger clinical responses. Further studies are needed to investigate such possible synergy in ALL cases with other mutations in the IL7R-JAK-STAT pathway.

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