Combination therapy of a PSEN1-selective γ-secretase inhibitor with dexamethasone and an XPO1 inhibitor to target T-cell acute lymphoblastic leukemia

γ-secretase inhibitors (GSI) are a potential therapeutic option for T-cell acute lymphoblastic leukemia (T-ALL) cases with a NOTCH1 mutation, but broad spectrum GSI cause severe gastro-intestinal toxicity.[1] We recently demonstrated that MRK-560, a PSEN1-selective GSI, is still active against leukemia cells and does not induce gastro-intestinal toxicity.[1] Here, we show that MRK-560 is synergistic with dexamethasone in T-ALL cell lines and that combination treatment prolongs survival in T-ALL patient-derived xenograft (PDX) mouse models. Moreover, we were able to further reduce leukemia development and prolong survival of T-ALL PDX mice by adding KPT-8602, an XPO1 inhibitor previously shown to have anti-leukemia activity in T-ALL.[2,3]

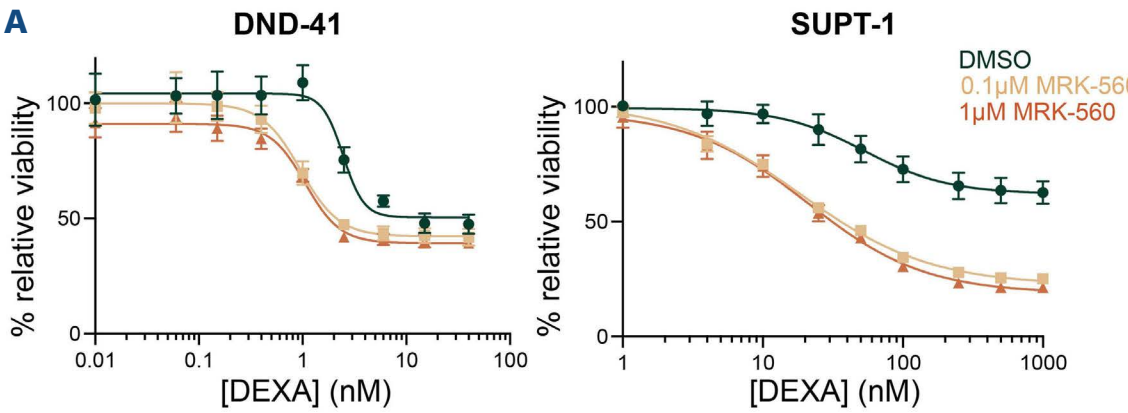
T-ALL is an aggressive hematological cancer, which arises from the accumulation of multiple genomic lesions in hematopoietic precursor cells.[4] Over the past years, the long-term survival of pediatric T-ALL has increased to almost 90% due to improved supportive care, optimization and intensification of multi-agent chemotherapy based on risk group stratification of patients.[5] Although this optimized therapy also improved the outcomes of adults, the long-term survival rate in this group hovers around 50%.[6] Moreover, the outcome remains poor for relapsed/refractory T-ALL and the side effects of chemotherapy, such as infections or cardiovascular impairment, also affect patients' health.[1,2,7] Therefore, there is a need for other targeted therapies, which are more efficient and less toxic compared to chemotherapy. One important therapeutic target for T-ALL is the NOTCH1 signaling pathway, since gain-of-function mutations in the NOTCH1 receptor are present in the majority of the patients.[4,8]

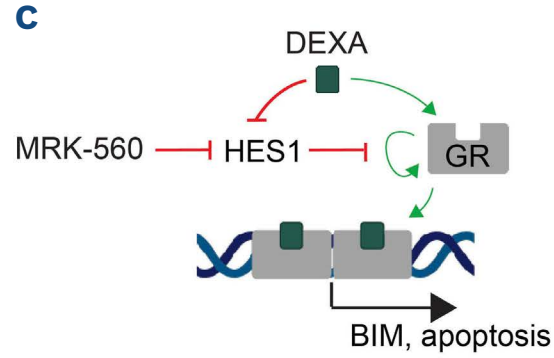
The transmembrane NOTCH1 receptor has to be cleaved by the γ-secretase complex in order to become active and to regulate gene expression of NOTCH1 downstream targets, such as *HES1*.[9] Broad-spectrum GSI effectively inhibit NOTCH1 cleavage by the γ-secretase complex and have been tested in clinical trials for the treatment of Alzheimer and T-ALL, showing on-target dose-limiting gastro-intestinal toxicities.[10,11] However, Habets *et al.* showed that T-ALL cells only contain PSEN1-containing γ-secretase complexes, while other cell types, such as intestinal cells, express both PSEN1 and PSEN2.[1] This explains the gastro-intestinal toxicity of broad-spectrum GSI and we recently demonstrated that MRK-560, a PSEN1-selective GSI, can be used to safely inhibit T-ALL cell growth in PDX, without severe gastro-intestinal toxicities.[1,2]

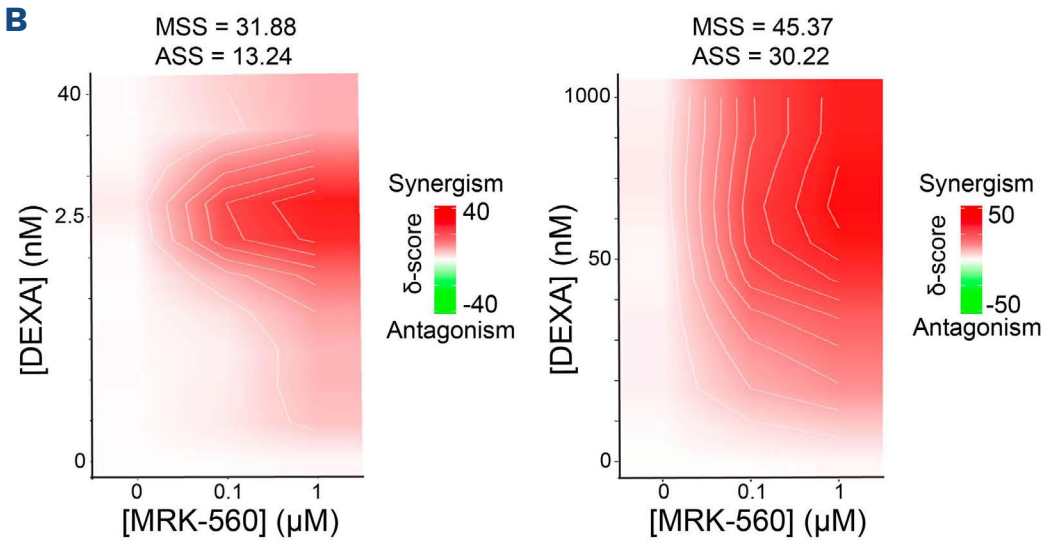
One important remaining aspect before using PSEN1-selective GSI in clinical trials is to investigate their combinatorial effect with currently used drugs, such as dexamethasone, doxorubicin and vincristine. In the current work, we tested the combination of the PSEN1-selective GSI, MRK-560, with currently used chemotherapeutic drugs in T-ALL cell lines and PDX models. After we obtained clear synergy between MRK-560 and dexamethasone, we also tested the triplet combination MRK-560, dexamethasone and KPT-8602. KPT-8602 is a second-generation XPO1 inhibitor, which showed potent activity against ALL and is currently tested in a clinical trial for multiple myeloma (*clinicaltrials gov. Identifier: NCT02649790*).[2,3,12]

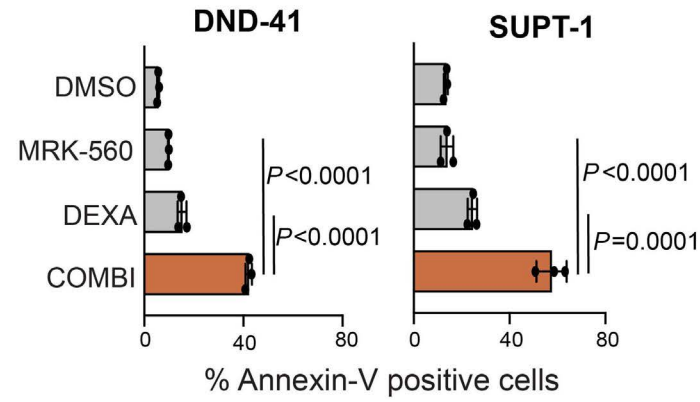
First, we determined the effect on cell viability of MRK-560 in combination with commonly used drugs by performing dose response curves in two T-ALL cell lines, DND-41 and SUPT-1 (Figure 1A; *Online Supplementary Figure S1B, C*). Both cell lines are sensitive to currently used drugs (dexamethasone, vincristine, doxorubicin) and are dependent on NOTCH1 signaling for survival and proliferation. DND-41 cells have a mutation in the heterodimerization and PEST domains of NOTCH1,[2] while SUPT-1 cells have a NOTCH1 translocation (t7;9)(q34;q34), resulting in a truncated NOTCH1 receptor, and both still require γ-secretase cleavage to activate the NOTCH1 mutants. The majority of T-ALL cell lines are not sensitive to dexamethasone and could therefore not be used in this study.

Treatment with MRK-560 increased the sensitivity of both NOTCH1-dependent cell lines to dexamethasone, with the largest effect observed in SUPT-1 cells (Figure 1A). In order to determine if MRK-560 and dexamethasone act synergistically, we calculated the synergy or δ score for each drug combination (Figure 1B). The average synergy score for SUPT-1 cells was higher compared to DND-41, which is in line with the dose response curves (Figure 1A). As control, we included a NOTCH1-independent dexamethasone-sensitive B-ALL cell line (697 cells) for which no major difference in dexamethasone sensitivity was observed between dimethyl sulfoxide (DMSO)- and MRK-560-treated cells (*Online Supplementary Figure S1A*), confirming the specificity of MRK-560 to NOTCH1. The same dose response curves and synergy plots were obtained for the combination between MRK-560 and other chemotherapeutic drugs (*Online Supplementary Figure*









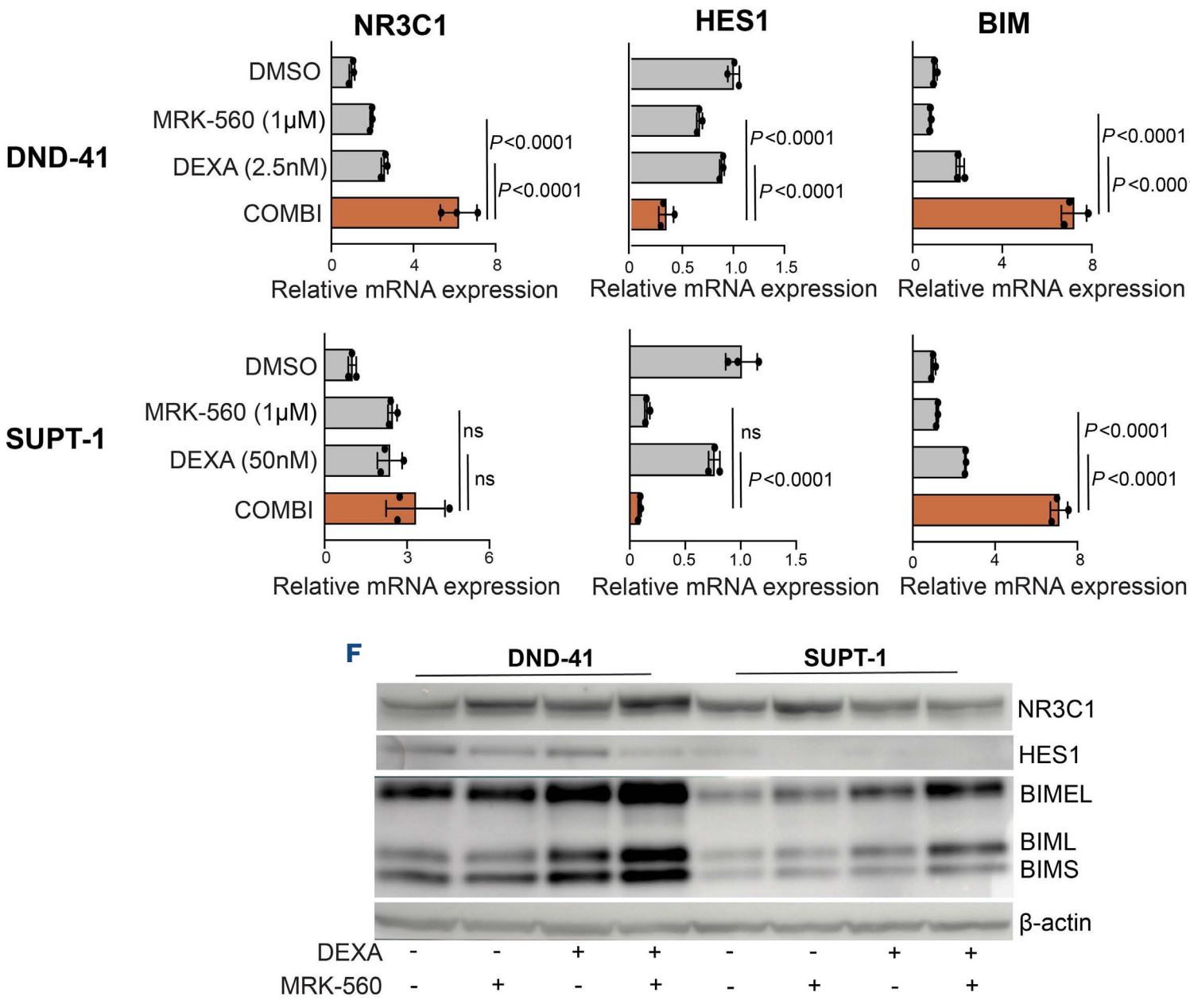


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Figure 1. MRK-560 synergizes with dexamethasone in T-cell acute lymphoblastic leukemia cell lines via HES1. The effect of MRK-560 treatment was visible after 5-7 days and therefore, cells were always pretreated with dimethyl sulfoxide (DMSO)/MRK-560 for 5 days, followed by 24 hours (h) (quantitative polymerase chain reaction, western blot) or 48 h treatment (dose response, apoptosis) with DMSO/MRK-560 alone or in combination with dexamethasone. (A) Dose response curves for 2 NOTCH1-dependent T-cell acute lymphoblastic leukemia (T-ALL) cell lines (DND-41 and SUPT-1). The relative viability was calculated based on the DMSO condition of each pretreatment group (DMSO/MRK-560). (B) Synergy plots representing the synergy or δ-score for each combination of MRK-560 and dexamethasone in DND-41 and SUPT-1 cells. (C) Synergy mechanism between MRK-560 and dexamethasone via HES1. Dexamethasone binds to and upregulates the glucocorticoid receptor (GR). Subsequently, BIM expression increases and results in apoptosis. Besides, HES1 is a suppressor of the GR auto-upregulation and is down regulated by γ-secretase inhibitors, such as MRK-560, which further increases the expression of BIM and apoptosis. (D) Annexin-V-positive cells for DND-41 and SUPT-1 cells after treatment of 48 h with/without highly synergistic concentration of dexamethasone (DND-41: 2.5 nM dexamethasone and 1 μM MRK-560, SUPT-1: 50 nM dexamethasone and 1 μM MRK-560). (E) mRNA expression levels of the glucocorticoid receptor (*NR3C1*), *HES1* and *BIM* after treatment of 24 h with/without highly synergistic concentration of dexamethasone. mRNA expression levels were normalized to housekeeping genes and the DMSO control. (F) Protein expression levels of glucocorticoid receptor (NR3C1), HES1 and BIM after treatment of 24 h with/without highly synergistic concentration of dexamethasone (DND-41: 2.5nM dexamethasone and 1 μM MRK-560, SUPT-1: 50 nM dexamethasone and 1 μM MRK-560). A loading control (β-actin) is included for all samples. All figures contain mean and standard deviation (error bars) of 3 replicates. Statistical differences were obtained after Sidak's multiple comparisons test (one-way ANOVA). MSS: maximum synergy score for a specific drug combination; ASS: average synergy score for all drug combinations. DEXA: dexamethasone.

S1B, C). For doxorubicin and vincristine, the overall synergy score suggested an additive effect between both drugs, rather than a synergistic effect. We also noticed that higher concentrations of doxorubicin and vincristine resulted in lower synergy scores. In agreement with this, another research group has found that HES1 is necessary for doxorubicin-driven apoptosis, which could explain the antagonistic effects at higher concentrations of doxorubicin.[13]

A potential synergy mechanism between NOTCH1 inhibition via broad spectrum GSI and glucocorticoids was already investigated previously (Figure 1C).[14,15] In order to evaluate if the same mechanism is valid for the combination between PSEN1-selective GSI MRK-560 and dexamethasone, we performed an apoptosis assay with annexin-V/PI staining (Figure 1D). Combination treatment significantly increased apoptosis by approximately 2.5-fold compared to dexamethasone-only treatment in both cell lines. We further investigated the synergy mechanism from Figure 1C by measuring gene expression and corresponding protein levels of glucocorticoid receptor (NR3C1), HES1 and BIM (Figure 1E, F). Combination treatment downregulated HES1 and increased NR3C1 and BIM mRNA and protein levels in both cell lines, which further confirmed the synergy mechanism from Figure 1C.

In order to study whether the observed synergy between MRK-560 and dexamethasone *in vitro* could also prolong survival in mice, we set up an *in vivo* experiment with the T-ALL PDX X10, which we engineered to express luciferase (Figure 2A; *Online Supplementary Table S1*).[12] Drug toxicity was evaluated based on weight changes during treatment and no significant differences were observed between vehicle and treated mice (Figure 2B). In addition, the increase in human CD45+ cells in peripheral blood after treatment was delayed in mice treated with the combination, compared to single treated mice (Figure 2B). Moreover, the bioluminescent images and the corresponding total fluxes of the different treatment groups also showed significant reduction in leukemia progression of combination *versus* single treatment (Figure 2C, D). These results indicate that combining MRK-560 and dexamethasone *in vivo* delay leukemia progression compared to MRK-560 or dexamethasone alone. This prolonged suppression of leukemia in combination-treated mice also resulted in a significant increase in survival compared to MRK-560 or dexamethasone alone (Figure 2E).

After showing synergy between MRK-560 and dexamethasone, we tested the triple combination of MRK-560, dexamethasone and the XPO1 inhibitor KPT-8602 in a second *in vivo* experiment (Figure 3A). The percentage of human CD45$^+$ cells in spleen and bone marrow was significantly lower in the triple-combination treatment (Figure 3B), indicating that the triple-combination treatment is more effective for T-ALL patients. Here, we did not observe significant weight changes between double- and triple-combination treatment (*Online Supplementary Figure S2A*). Furthermore, we did not observe increased goblet cell counts in the gastro-intestinal tract or macroscopic skin lesions for any of the treatments, but there was a significant decrease in skin thickness after drug treatment compared to vehicle (Figure 3C, D). Overall, these results confirm a low toxicity of MRK-560 in combination with dexamethasone or KPT-8602.

Since we did not detect severe toxicity with double- or triple-combination treatment, we next performed more *in vivo* experiments to obtain preclinical data in five T-ALL PDX models (XB41, X12, X14, X09, XC63) with different NOTCH1 and/or FBXW7 mutations (Figure 3E; *Online Supplementary Table S1*). In placebo-treated mice, human leukemia cells (detected by anti-human CD45) represented >50% of peripheral white blood cells in less than 20 days, while dual combination of drugs delayed this to over 40 days and the triple combination to about 70 days (*Online Supplementary Figure S2B*). These effects were

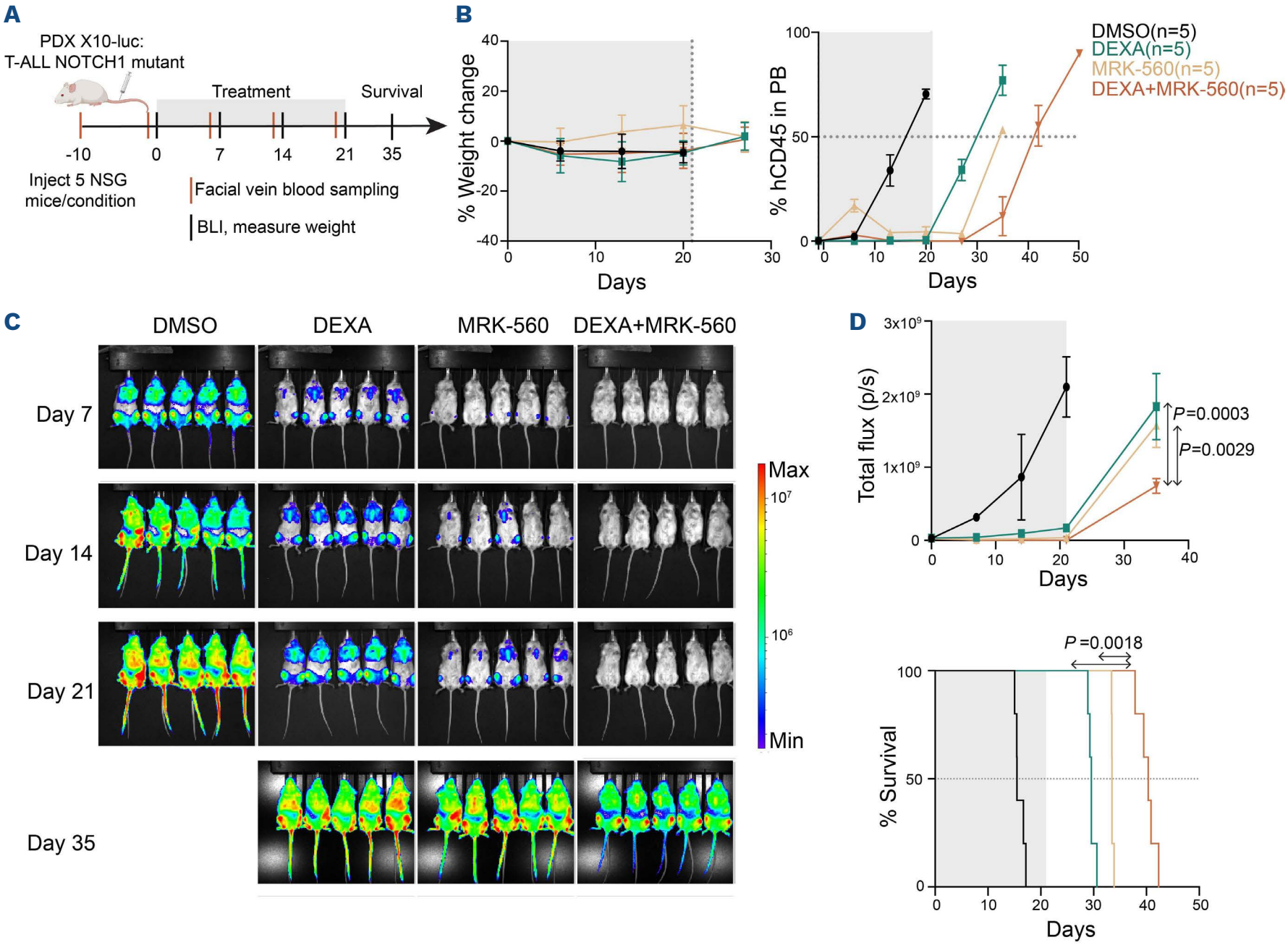

Figure 2. Combination treatment with MRK-560 and dexamethasone results in a survival benefit in T-cell acute lymphoblastic leukemia patient-derived xenograft mice. (A) Schematic representation of the set-up of the *in vivo* experiment with patient-derived xenograft (PDX) X10-luciferase (X10-luc) sample. Twenty female immunodeficient mice (8-10 weeks old) were injected with X10-luc cells and treatment with vehicle (n=5), MRK-560 (15 mg/kg, intraperitoneally [IP], n=5), dexamethasone (5 mg/kg, IP, n=5) or the combination (n=5) was started after engraftment (0.5-1% human CD45 in blood). After treatment, mice were subjected to survival analysis and were marked as 'death to leukemia' when they reached 50% human CD45 in blood. (B) Percentage of weight changes compared to initial weight at start of treatment and percentage of human CD45$^+$ cells in peripheral blood (PB) of all treatment groups at different time points. (C) Normalized bioluminescent images of each treatment group at different time points during treatment (day 7, 14 and 21) and after treatment (day 35). In order to obtain bioluminescent imaging (BLI) images, mice were anesthetized with 2% isoflurane, injected subcutaneously with D-luciferin (126 mg/kg) and imaged with the IVIS Spectrum. (D) Maximum total flux (photons/second) of the BLI figures. Statistical analysis of the maximum total flux at day 35 was performed with one-way ANOVA Sidak's multiple comparison test. (E) Kaplan-Meier survival plots for the different treatment groups with X10-luc. The data were analyzed using log-rank Mantel-Cox statistical test. The grey color in all figures represents the treatment period. All figures show mean and standard deviation (error bars) of 5 mice (5 mice/treatment group).DEXA: dexamethasone; DMSO: dimethyl sulfoxide.

obtained with only 3 weeks of treatment. Data for MRK-560 plus KPT-8602 were re-used from a previous study using the same PDX models.[2] Separate graphs for each PDX sample are presented in the *Online Supplementary Figure S2C*. We also have to point out here that toxicity monitoring by weight of the mice did indicate significant weight loss associated with the triple-combination treatment, which requires further monitoring of possible toxicity with this treatment (*Online Supplementary Figure S2B*). Overall, we conclude that treatment with MRK-560 and dexamethasone prolongs survival with 7 days compared to dexamethasone only, while the triple-treatment combination significantly increased survival with 45 or 28 days compared to vehicle or dexamethasone-only treatment, respectively (Figure 3F).

In conclusion, we demonstrate that the combination between MRK-560 and dexamethasone, a cornerstone in the current treatment of T-ALL, is synergistic and that the underlying mechanism between both drugs is comparable to previously described between broad-spectrum GSI and

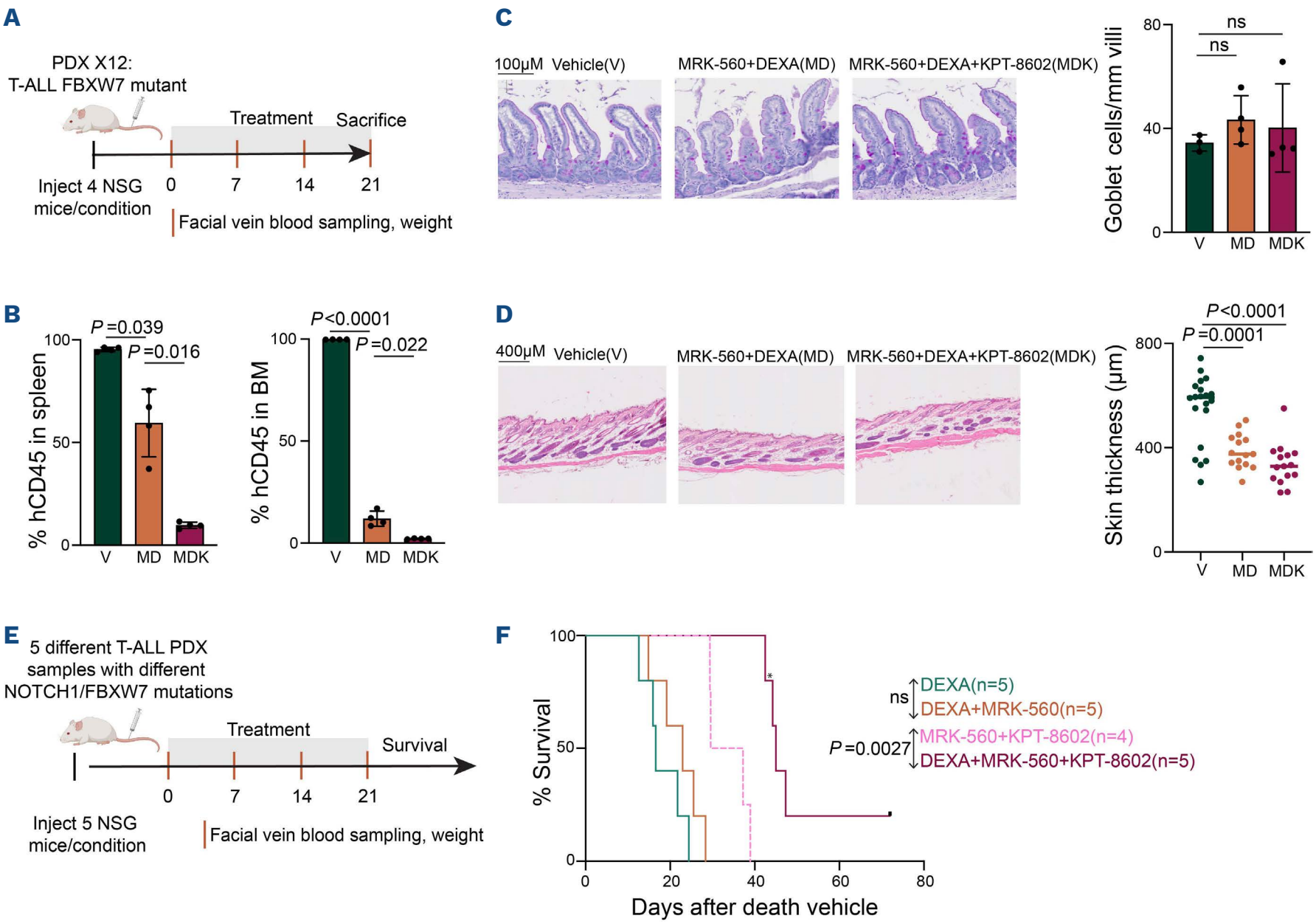

Figure 3. Treatment with MRK-560/dexamethasone/KPT-8602 combination further prolongs T-cell acute lymphoblastic leukemia survival. (A) Schematic representation of the set-up of the *in vivo* experiment with X12 patient-derived xenograf (PDX). Twelve female immunodeficient mice (8-10 weeks old) were injected with X12 cells and treated with vehicle (n=4), double combination (MRK-560: 15 mg/kg, intraperitoneally [IP] – dexamethasone: 5 mg/kg, IP) (n=4) or the triple combination (KPT-8602: 5 mg/kg, oral gavage) (n=4) after engraftment. After 3-week treatment (5 days on – 2 days off), all mice were sacrificed. (B) Percentage of human CD45$^+$ cells in spleen and bone marrow (BM) after 3-week treatment. (C) Images of periodic Acid-Schiff staining on intestines together with the amount of goblet cells per millimeter of villus after 3-week treatment. Scale bar, 100 µM. (D) Images of hematoxylin and eosin staining of skin section of the back of mice together with skin thickness after 3-week treatment. For each mouse, the skin thickness was measured at 5 different locations and all data points are given in this figure. Scale bar, 400 µM. (E) Schematic representation of the set-up of the *in vivo* experiment with different PDX models: X12, X14, XB41, X09, XC63. For each PDX sample, 5 mice were injected and divided into the 5 treatment groups after engraftment. After 3 weeks of treatment (5 days on – 2 days off) with vehicle, MRK-560 (15 mg/kg, IP), dexamethasone (5 mg/kg, IP), MRK-560 + dexamethasone or MRK-560 + dexamethasone + KPT-9602 (KPT-8602: 5 mg/kg, oral gavage) mice were subjected to survival analysis and were marked as 'death to leukemia' when blood human CD45 levels reached 50%. (F) Kaplan-Meier survival plots, normalized to death of vehicle. This graph also include data from Govaerts *et al.* since the same PDX samples were used:[2] vehicle and MRK-560 survival values are the average of this experiment and the ones obtained in Govaerts *et al.*, MRK-560 and KPT-8602 combination curve was copied from Govaerts *et al.* The data were analyzed using log-rank Mantel-Cox statistical test. *XC63 mouse was treated with 2.5 mg/kg KPT-8602 instead of 5 mg/kg. The experiment was stopped after 98 days, the last triplet combination mouse was sacrificed and leukemia was detected in bone marrow. The grey color in all figures represent the treatment period. All graphs show mean and standard deviation (error bars). DEXA: dexamethasone; T-ALL: T-cell acute lymphoblastic leukemia; NSG: immune deficient mice (NSG strain), ns: not significant.

dexamethasone.[14] Furthermore, other studies showed that co-treatment between broad-spectrum GSI and glucocorticoids can reverse the gastro-intestinal toxicity observed with these GSI and can even reverse glucocorticoid resistance.[14,15] All these data suggest that MRK-560 can be safely combined with dexamethasone for the treatment for T-ALL by increasing apoptosis in leukemia cells and by restoring dexamethasone sensitivity in resistant patients. We also showed that we can further increase survival in PDX models with different NOTCH1/FBXW7 mutations after treatment with the triple-combination therapy with MRK-560, dexamethasone and the XPO1 inhibitor KPT-8602. Importantly, we observed that treatment with double- or triple-combination did not show severe toxicity on the

gastro-intestinal system. Future clinical trials are needed to determine if such drug combinations are beneficial to T-ALL patients with sub-optimal responses to chemotherapy, or if the inclusion of these targeted drugs can reduce the dose of the chemotherapeutic compounds and limit their side effects. Additionally, relapse or dexamethasone-resistant patients can benefit from this combination treatment since MRK-560 can increase the sensitivity of leukemia cells to dexamethasone.

Authors

Charlien Vandersmissen,[1,2,3] Cristina Prieto,[1,2,3] Olga Gielen,[1,2,3] Kris Jacobs,[1,2,3] David Nittner,[2] Johan Maertens,[3,4,5] Heidi Segers[3,6,7] and Jan Cools[1,2,3]

[1]Center for Human Genetics, KU Leuven; [2]Center for Cancer Biology, VIB; [3]Leuvens Kanker Instituut (LKI), KU Leuven – UZ Leuven; [4]Department of Hematology, UZ Leuven; [5]Department of Microbiology, Immunology and Transplantation, KU Leuven; [6]Department of Oncology, KU Leuven and [7]Department of Pediatric Oncology, UZ Leuven, Leuven, Belgium

Correspondence:
J. COOLS - jan.cools@kuleuven.be

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Disclosures

No conflicts of interest to disclose.

Contributions

CV designed the study and developed the methodology, performed experiments, analyzed data and wrote the original draft. CP developed the methodology, supervised the study and wrote the original draft. OG, KJ and DN performed experiments. JM and HS collected human T-ALL samples. JC designed the study and methodology, supervised the study and wrote the original draft.

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Data-sharing statement

The data is available by contacting the corresponding author.

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