Supplementary Material

Safe targeting of T cell acute lymphoblastic leukemia by pathology-specific NOTCH inhibition

Roger A. Habets, Charles E. de Bock, Lutgarde Serneels, Inge Lodewijkx, Delphine Verbeke, David Nittner, Rajeshwar Narlawar, Sofie Demeyer, James Dooley, Adrian Liston, Tom Taghon, Jan Cools and Bart de Strooper

Supplementary Materials and Methods

Chemistry. MRK-560 (N-[4-(4-chlorophenyl)sulfonyl-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoro methanesulfonamide) was kindly provided by Janssen Pharmaceutica and synthesized as follows. 4-(4-chlorophenyl)sulfonyl-4-(2,5-difluorophenyl)cyclohexanamine was synthesized using the literature procedure (61). Trifluoromethanesulfonic anhydride (0.214 ml, 1.269 mmol) was added to a stirred solution of 4-(4-chlorophenyl)sulfonyl-4-(2,5-difluorophenyl)cyclohexanamine (200 mg, 0.518 mmol) and triethyl amine (0.177 ml, 1.273 mmol) in anhydrous dichloromethane (10 ml) at 0 °C and the reaction mixture was stirred further for 90 min under inert atmosphere. Upon completion (Thin Layer Chromatography), the reaction mixture was warmed to ambient temperature and concentrated in vacuo. The residue was then diluted with ethyl acetate (50 ml), washed with 2N NaOH, water, brine, dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo. The crude compound was purified by flash column chromatography using 15% ethyl acetate in n-heptane as eluent to afford the desired compound as colorless solid (215 mg, 80%). 1H NMR (400 MHz, DMSO-d6): 1.49 (br t, J=13.8 Hz, 2 H), 1.82 (br d, J=14.2 Hz, 2 H), 2.33 - 2.46 (m, 2 H), 2.52 - 2.62 (m, 2 H), 3.61 - 3.67 (m, 1 H), 7.11 - 7.24 (m, 2 H), 7.29 - 7.34 (m, 1 H), 7.34 - 7.39 (m, 2 H), 7.61 - 7.65 (m, 2 H), 9.76 (br d, J=5.3 Hz, 1 H) δ ppm. 13C NMR (100 MHz, DMSO-d6): 23.94 (br d, J=5.1 Hz, 1 C), 27.61 (br s, 1 C), 49.14 (s, 1 C), 69.90 (d, J=4.4 Hz, 1 C), 117.89 - 118.41 (m, 1 C), 118.42 - 118.76 (m, 1 C), 118.97 (dd, J=26.0, 4.0 Hz, 1 C), 120.76 (dd, J=11.7, 7.3 Hz, 1 C), 129.09 (s, 1 C), 131.66 (s, 1 C), 133.23 (s, 1 C), 139.56 (s, 1 C), 156.66 - 157.52 (m, 1 C), 159.54 (dd, J=62.7, 1.8 Hz, 1 C) δ ppm. 19F NMR (377 MHz, DMSO-d6): 117.99 (s, 1 F), -113.25 (s, 1 F), -77.39 (s, 1 F) δ ppm. LCMS (ESI): 535.1 [M+NH4]+, 516.1 [M-H]- Purity: 99% (retention time: 2.20 min).

Antibodies for Western blotting. Rabbit polyclonal antibodies against APH1A (B80.3), APH1B (B78.2), NCT (9C3) and PEN-2 (B126) were described previously *(66, 67)*. Rabbit anti-Cleaved-NOTCH1-Val1744 (D3B8), anti-PSEN1-CTF (D39D1) and anti-PSEN2-CTF (D30G3) were purchased from Cell Signaling, Mouse anti-beta-actin (A5441) from Sigma and Rabbit anti-tRFP (AB234) was

from Evrogen. Secondary antibodies were conjugated with horseradish peroxidase and bands were visualized using a cooled charge-coupled device camera (ImageQuant LAS-4000; GE Healthcare).

Western blotting. MEFs, T-ALL cell lines or single cell preparation from mouse tissues were lysed in STE buffer (250 mM sucrose, 5 mM Tris pH7, 1 mM EGTA) + 1% TX-100 and incubated on ice for 30 min. The supernatant was collected after centrifugation at 19,000 *g* for 30 min at 4 °C. Equal amounts of protein were separated on NuPAGE NOVEX Bis-Tris gels (Life Technologies).

Presenilin selectivity assay. MEFs cells expressing the specified γ-secretase complexes were treated with the different compounds as indicated for 18 h in DMEM/F12 supplemented with 2% FBS.

Ex vivo T cell cultures. Bone marrow cells were isolated from femur and tibia. Lineage-negative cells were enriched by negative selection using biotinylated antibodies directed against non-hematopoietic stem cells and non-progenitor cells (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119, 7-4) and streptavidin-coated magnetic particles (RapidSpheres, STEMCELL Technologies). Enriched cells were cultured on DLL4-Fc coated plates in the presence of mIL-7 (20 ng/ml) and mSCF (20 ng/ml). After differentiation into DN2 stage pro-T cells ($CD44^{+}CD25^{+}$), cells were transduced with MSCV-NOTCH1-L1601P-ΔP-IRES-GFP, and GFP-positive cells were sorted by FACS (S3 cell sorter, Bio Rad). To assess processing and signaling from transduced mutant NOTCH proteins, cells were cultured in the absence of DLL4 during the course of experiments unless stated otherwise.

Immunohistochemistry. Tissues were collected and fixed in 10% neutral buffered formalin (Sigma) for 48 hours and then processed for paraffin embedding (HistoStar Embedding Workstation). Sections of 7 µm of thickness obtained from the paraffin-embedded tissues (Thermo Scientific Microm HM355S microtome) were mounted on Superfrost Ultra Plus Adhesion slides (Thermo Scientific) and stained with hematoxylin and eosin (Mayers Haematoxylin 1l, Leica, 3801582E and Eosin Y solution, aqueous (1 liter), Sigma-Aldrich, HT110232-1L) for histopathological examination. Sections were then stained with periodic acid–Schiff (PAS): Slides were incubated in freshly prepared periodic acid (0.5 %) for 15 min and rinsed and incubated for another 5 min in distilled water. The Schiff reagent (Sigma-Aldrich, 3952016-500ML) was added onto the slides and kept for 15 min at room temperature in dark. The sections were then washed in lukewarm running tap water for 5 min, rinsed and incubated in distilled water for 2 min, and counterstained in Mayer's hematoxylin (Mayers Haematoxylin 1l, Leica, 3801582E) for 1 min. After washing in running tap water for 5 min sections were dehydrated (95% EtOH, 100% EtOH, 100% EtOH, 3 min each, followed by two times xylene for 5 min) and mounted in DPX mounting medium (Sigma, 06522). Images were acquired on the Zeiss Axio Scan.Z1 using a 20x objective and ZEN 2 software. For exporting images, we used the ZEN 2 software (Zeiss).

Immunofluorescence with tyramide signal amplification. The following antibodies were used for detecting the respective proteins: anti-Ki67 (rabbit, 1:1000, ThermoScientific, RM-9106-S, Clone SP6), anti-HLA-A (rabbit, 1:1250, Abcam, ab52922). Furthermore, the PerkinElmer Opal 4-Color Manual IHC Kit (PerkinElmer, NEL810001KT) was used for the tyramide signal amplification according to the manufacturer's protocol. To introduce the secondary-HRP, we used the Envision+/HRP goat anti-Rabbit (Dako Envision+ Single Reagents, HRP, Rabbit, Code K4003) for antibodies raised in rabbit. The various proteins were detected using the OPAL 520 for ki67 and OPAL 690 for HLA-A. Images were acquired on the Zeiss Axio Scan.Z1 using a x20 objective and ZEN 2 software. To export images, we used the ZEN 2 software (Zeiss) and the software package QuPath Version: 0.1.2 (*(68);* https://github.com/qupath/qupath/wiki/Citing-QuPath). QuPath was also used for automatic cell detection using the DAPI channel and for subsequent creation of a detection classifier using all 55 given parameters resulting in the classification of Ki67 and HLA-A-double positive cells within the subpopulation of all HLA-A positive cells in the whole slide.

Flow cytometric analysis of hematopoietic populations. Single-cell suspensions were prepared from peripheral blood, bone marrow, spleen, thymus and lymph nodes. Single cells were stained with antibodies against CD4-eFluor 450 (eBioscience), CD4-PECy7 (eBioscience) or CD4-PE (eBioscience), CD8-PerCp-eFluor 710 (eBioscience), CD8-APC-eFluor 780 (eBioscience) or CD8-APC (eBioscience), CD25-Alexa-488 (eBioscience) or CD25-APC (BD Pharmingen), CD44-Brilliant Violet 510 (Biolegend) or CD44-PerCpCy5 (eBioscience) and hCD45-APC (eBioscience), as indicated and analyzed on a FACSCanto flow cytometer (BD Biosciences) or a MACSQuant VYB cell cytometer (Miltenyl Biotec). Data were analyzed using FlowJo software (Tree star).

Quantitative real-time PCR. RNA was extracted from tissue and cells using the illustra RNAspin Mini Kit (GE Healthcare Life Sciences) as per manufacturer's instructions. cDNA synthesis was carried out using GoScript (Promega) and real time quantitative PCR performed using the GoTaq qPCR master mix (Promega) with the ViiA7 Real Time PCR system (Applied Biosystem). Quality control and analysis of data and primer efficiency were carried out using qbase+ software (Biogazelle). All gene expression was normalized using two housekeeping reference genes. Primers used for qPCR are listed in table S2.

Proliferation assays and cell cycle analysis. T-ALL cell lines HPB-ALL, DND-41 and Jurkat were treated with the γ-secretase inhibitors DAPT and MRK-560 as indicated for 14 days. Cells were subcultured every 2-3 days by centrifugation and resuspension in fresh medium containing the indicated compounds, as described previously *(39)*. Total cell numbers and cell viability were determined by measuring FSC/SSC on a MACSQuant VYB cell cytometer (Miltenyl Biotec). Cell cycle assays were performed with the Click-iT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermofisher Scientific) as per manufacturer's instructions. Briefly, 400 000 cells/ml were seeded and treated with

DMSO or 100, 300, and 1000 nM MRK-560 for 5 days. Two hours before the indicated time point, the cells were pulsed with 10 µM EdU. Cells were collected, washed and fixed. The FxCycle Violet Ready Flow DNA stain (Thermofisher scientific) was added, followed by 30 min incubation time. Stained cells were analyzed on the MACSQuant Vyb (Miltenyi). Data analysis was performed using FlowJo software (Tree Star).

Fig. S1

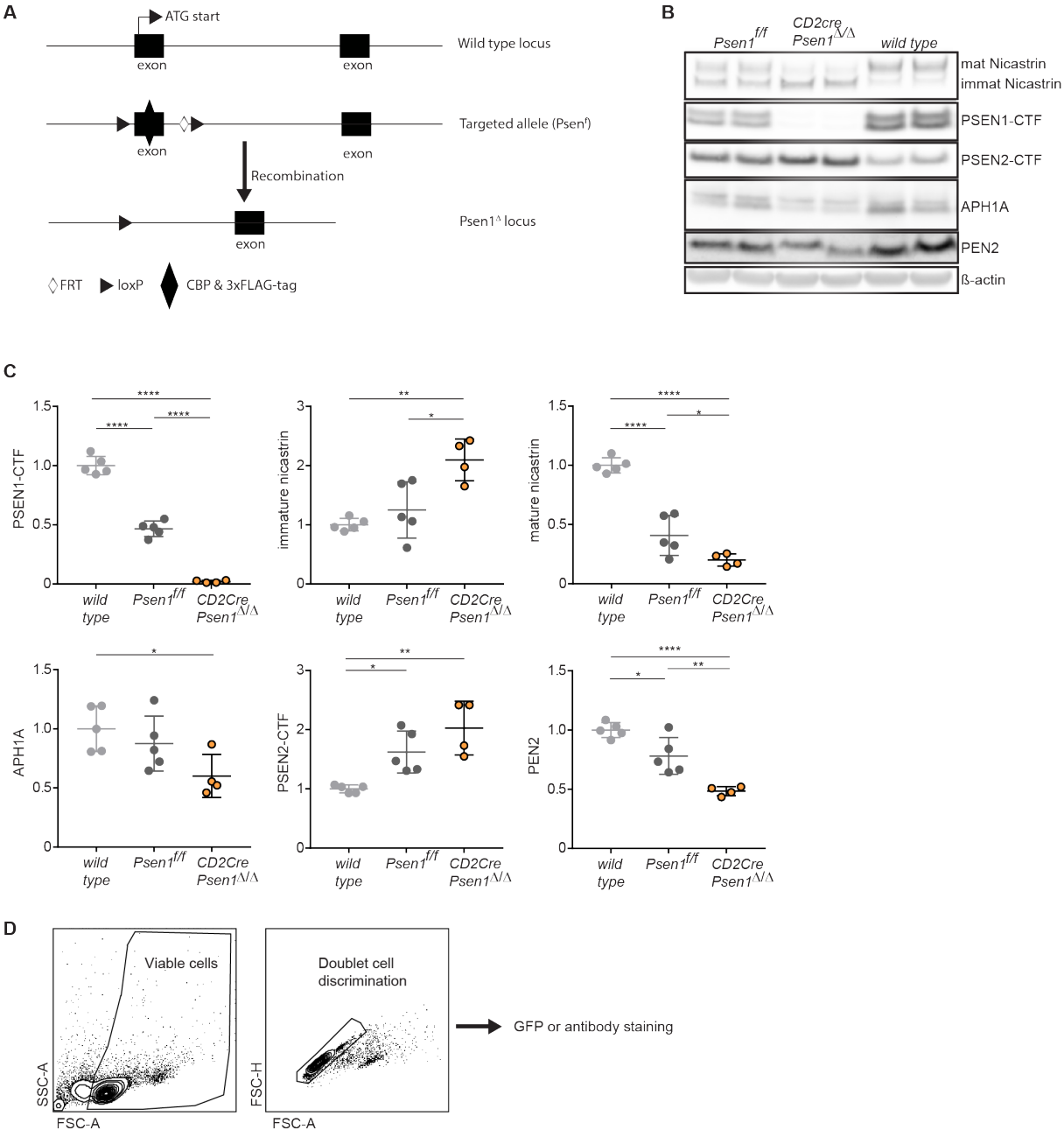

Fig. S1. PSEN1 deletion does not affect T-cell development.

(**A**) Generation of PSEN1 conditional knockout mice. (**B**) Representative western blot analysis of total thymus of C57BL/6 wildtype, *Psen1$^{f/f}$*, or *CD2CrePsen1$^{\Delta/\Delta}$* mice for the different γ-secretase subunits,

nicastrin, PSEN1, PSEN2, APH1A, PEN2, and β-actin. (**C**) Quantification of protein levels of gamma-secretase subunits from **B**. (**D**) Gating strategy used for all flow cytometry plots depicted in the manuscript. First, cells were gated according to physical parameters (FSC-A/SSC-A) to discard cell debris and residual red blood cells. Next, cell singlets were gated (FSC-A/FSC-H) to discard clumps and doublets. These populations were then either assessed for GFP expression or stained with the appropriate antibodies as described. All graphs show the mean values, and error bars represent standard deviation. P values in **C** were calculated using one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$ and **** $P \leq 0.0001$.

Fig. S2

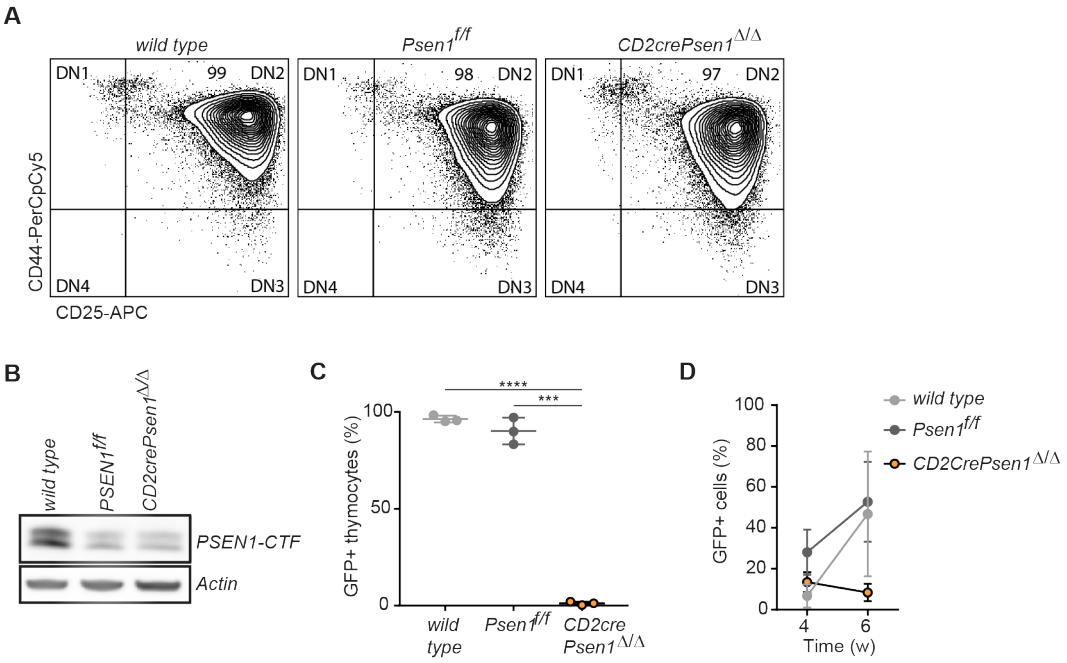

Fig. S2. PSEN1 deletion does not affect engraftment in bone marrow transplants.
(**A**) Representative flow cytometry plots of ex vivo cultures of mouse pro-T cells derived from C57BL/6 wild type, *Psen1*$^{f/f}$, or *CD2CrePsen1*$^{\Delta/\Delta}$ mice and stained with antibodies to CD44 and CD25. (**B**) Western blot analysis for PSEN1 and β-actin in enriched hematopoietic stem and progenitor cells isolated from C57BL/6 wildtype, *Psen1*$^{f/f}$, or *CD2CrePsen1*$^{\Delta/\Delta}$ donor mice used for transplantation. (**C**) Quantification of GFP^{+} cells in thymi from mice transplanted with wildtype, Psen1$^{f/f}$ or CD2CrePsen1$^{\Delta/\Delta}$ progenitors expressing ΔEGF-NOTCH1-L1601P-ΔP 9 weeks after transplant. (**D**) Quantification of circulating GFP^{+} cells in peripheral blood from mice transplanted with wildtype, *Psen1*$^{f/f}$, or *CD2CrePsen1*$^{\Delta/\Delta}$ progenitors expressing ΔEGF-NOTCH1-L1601P-ΔP 4 and 6 weeks after transplant. All graphs show the mean values, and error bars represent standard deviation. The P values in **C** were calculated using one-way ANOVA. *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Fig. S3

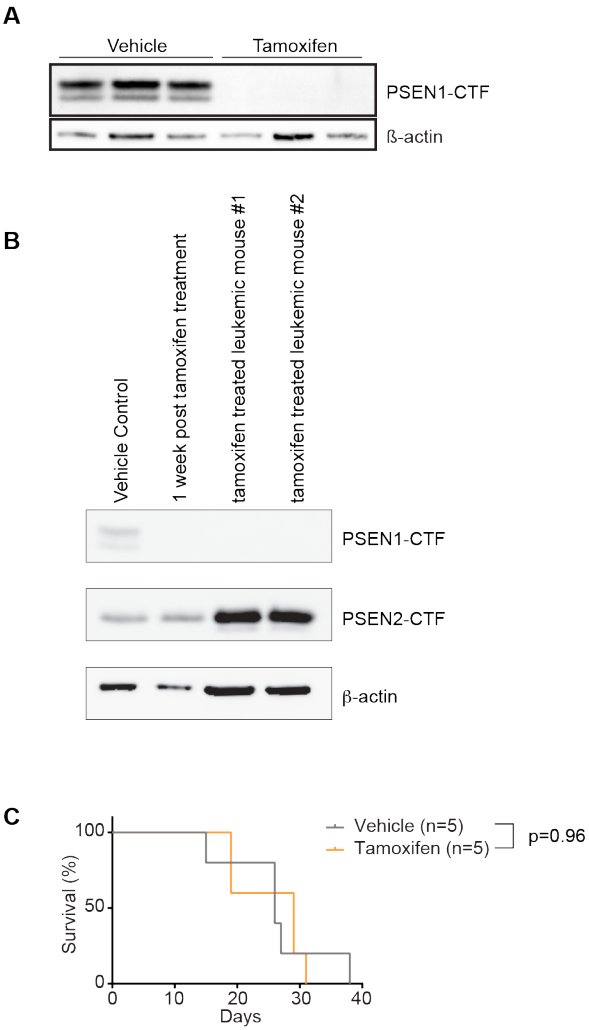

Fig. S3. Tamoxifen does not affect leukemia progression.

(**A**) Western blot analysis for PSEN1 and β-actin in leukemic cells recovered from spleens ~2 weeks after tamoxifen or vehicle treatment from mice transplanted with *R26Cre-ER^{T2}Psen1$^{f/f}$* progenitors expressing ΔEGF-NOTCH1-L1601P-ΔP. (**B**) Western blot analysis for PSEN1, PSEN2, and β-actin in leukemic cells recovered from a vehicle-treated spleen, a spleen after ~1 week of tamoxifen treatment, and two mice that developed leukemia after tamoxifen treatment following transplantation with *R26Cre-ER^{T2}Psen1$^{f/f}$* progenitors expressing ΔEGF-NOTCH1-L1601P-ΔP. (**C**) Kaplan-Meier survival curves of mice transplanted with C57BL/6 wildtype progenitors expressing ΔEGF-NOTCH1-L1601P-ΔP and treated with either vehicle or tamoxifen (100 mg/kg/day tamoxifen by I.P. injection on 5 consecutive days).The P values in **C** were calculated using the log-rank test.

Fig. S4

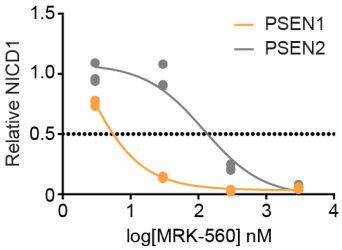

Fig. S4. MRK-560 shows selectivity for PSEN1 over PSEN2.

Quantification of NICD1 generation in MEFs expressing PSEN1 or PSEN2 γ-secretase complexes in response to MRK-560 treatment (n=3).

Fig. S5

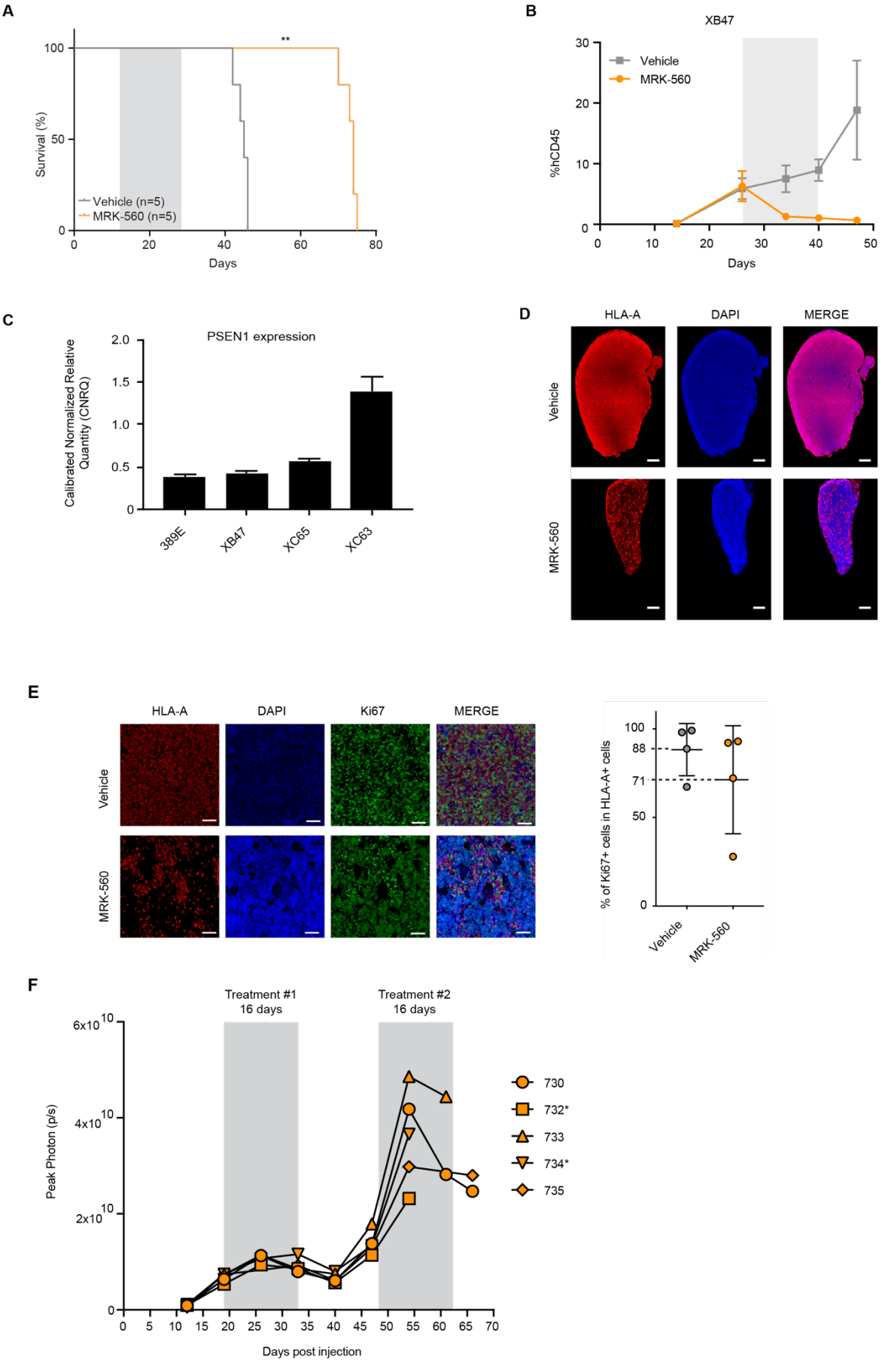
A
Survival (%)
Days
Vehicle (n=5)
MRK-560 (n=5)
**
B
XB47
Vehicle
MRK-560
%hCD45
Days
C
PSEN1 expression
Calibrated Normalized Relative Quantity (CNRQ)
389E
XB47
XC65
XC63
D
HLA-A
DAPI
MERGE
Vehicle
MRK-560
E
HLA-A
DAPI
Ki67
MERGE
Vehicle
MRK-560
% of Ki67+ cells in HLA-A+ cells
F
Treatment #1
16 days
Treatment #2
16 days
Peak Photon (p/s)
Days post injection
730
732*
733
734*
735

Fig. S5. Treatment of patient-derived xenograft samples with MRK-560.
(A) Kaplan-Meier survival curves of mice transplanted with patient sample XC65 and treated with vehicle or MRK-560 for 14 days. Treatment was started when 1% hCD45+ cells were detected in the blood. **(B)** Leukemia burden assessed by human CD45 staining of peripheral blood after 14 days of treatment with 30 μmol/kg MRK-560 or vehicle by subcutaneous injection (gray box) in PDX XB47. **(C)** Quantitative real time PCR analysis of *PSEN1* expression. **(D)** Human HLA-A immunofluorescent staining of spleen sections from PDX XC63 obtained immediately after 14 days of treatment with 30 μmol/kg MRK-560 or vehicle by subcutaneous injection (scale bar = 500 μm). **(E)** Representative higher magnification of human HLA-A and Ki67 immunofluorescent staining of spleen sections from PDX XC63 obtained immediately after 14 days of treatment with 30 μmol/kg MRK-560 or vehicle by subcutaneous injection. Associated quantification of Ki67 in HLA-A positive cells comparing MRK-560 (n=4) and vehicle (n=4) treated mice (scale bar = 50 μm). **(F)** Leukemia burden assessed by bioluminescence after two rounds of 30 μmol/kg MRK-560 treatment of PDX XC65, with the second round of treatment begun during relapse to determine whether the sensitivity to MRK-560 was maintained.

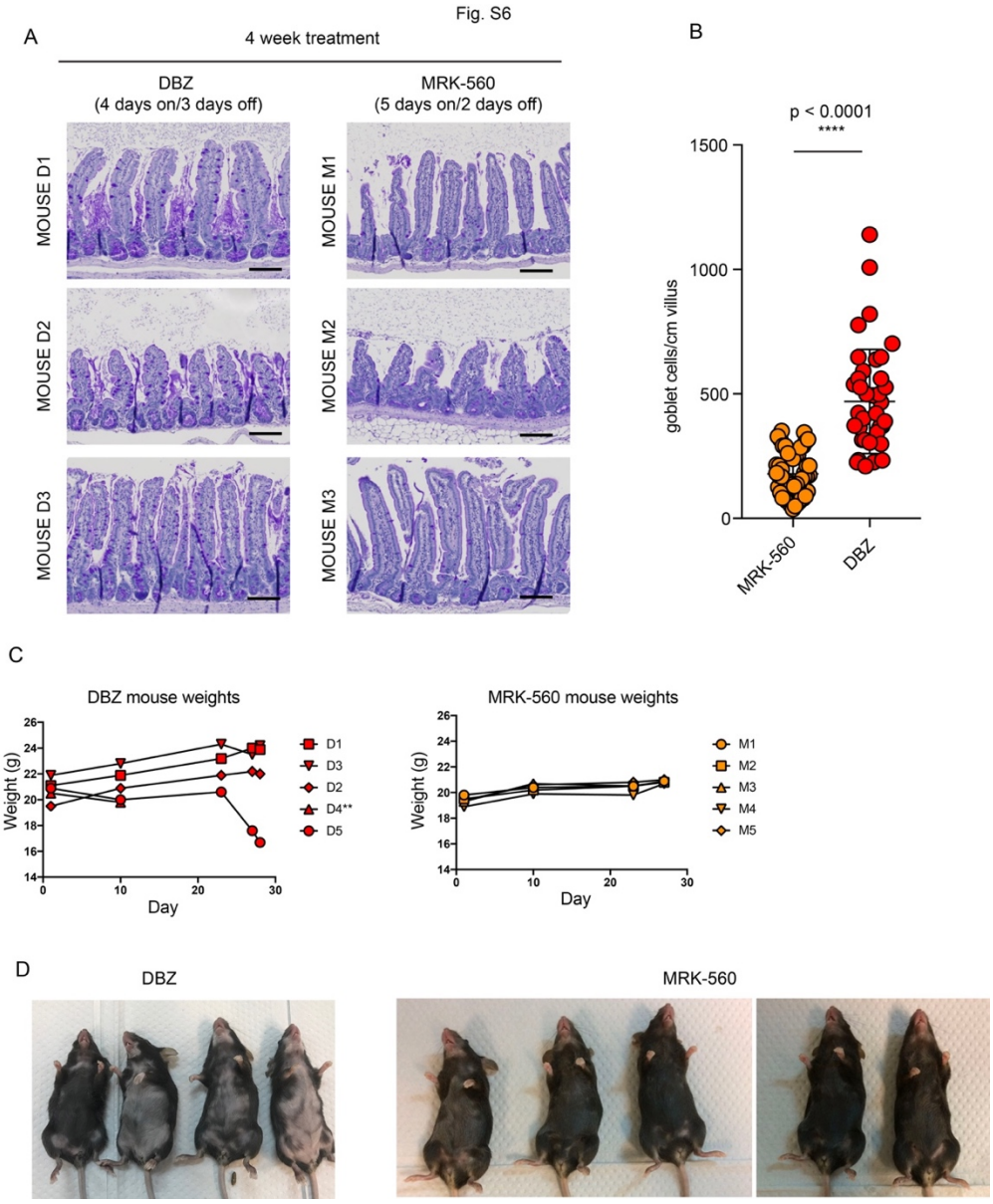

Fig. S6. Long term treatment with MRK-560 has less gastrointestinal toxicity compared to DBZ. **(A)** Representative images of periodic acid-Schiff staining of intestines from mice treated with MRK-560 or DBZ for 4 weeks to assess the number of secretory goblet cells. Scale bars represent 100 μm. **(B)** Quantification of the number of goblet cells per cm villus with 10 villi counted per mouse (MRK-560 n=5 and DBZ n=4). **(C)** Weights of mice over the 4-week treatment period**. (D)** Visible hair loss in DBZ-treated mice compared to MRK-560-treated mice at the end of the 4-week treatment period.

Table S1. T-ALL cell lines and patient-derived xenograft models.

	Mutations	**Subtype**
Cell lines		
HPB-ALL	*NOTCH1(L1575P), NOTCH1 PEST indel FBXW7(R347H), WT1(S189*), TP53(R141C)*	*TLX3*
DND-41	*NOTCH1(L1594P), NOTCH1(D1610V), NOTCH1 PEST indel, IL7R indel, NRAS(Q61H)*	*TLX3*
Jurkat	*PTEN deletion, TP53 R64*, NOTCH1 ins1740QAVEPPPPAQLHFMYVA*	*TAL1*
PDX samples		
XC63	*NOTCH1*(L1678P), NOTCH1(Q2459*), *JAK3*(M511I)	*immature*
XC65	*NOTCH1*(A2438fs*), *JAK1* (R724H)	*immature*
389E	*NOTCH1*(L1600P), NOTCH1(P2514fs*), *JAK3*(M511I), *DNM2*	*immature*
XB47	*NOTCH1(L1600P), NOTCH1(Y2490*), RPL10(R98S), CNOT3(R745ins), del(9)(p21p21)*	*TAL1*

Table S2. Primers used for qPCR.

Gene	**Forward**	**Reverse**
DTX1	CGCAAGACCAAGAAGAAGC	CTCATCAGGTGGGTTTTTCAC
MYC	CTCGGATTCTCTGCTCTCCT	TTTCCACAGAAACAACATCG
NOTCH3	TTACGACTGTGCCTGTCTTCC	TATAGGTGTTGACGCCATCC
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
TBP	CGGCTGTTTAACTTCGCTTC	CACACGCCAAGAAACAGTGA