

Supplemental Information

Blockade of Oncogenic NOTCH1 with the SERCA Inhibitor CAD204520 in T Cell Acute Lymphoblastic Leukemia

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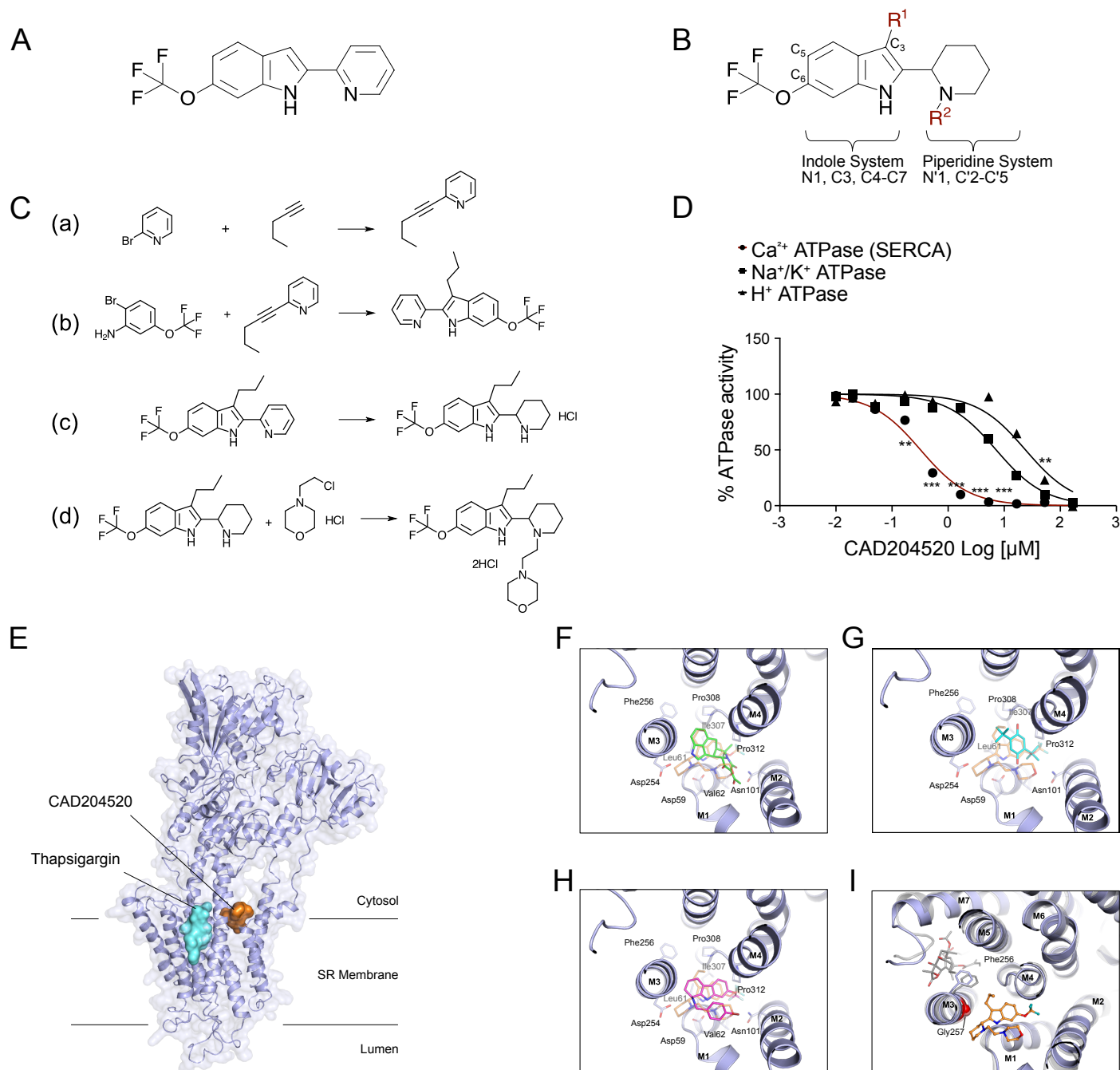


Figure S1 (Related to Figure 1 and Table S1): Synthesis route, activity and binding mode of CAD204520

A Chemical structure of the initial hit compound: 2-(2-pyridyl)-6-(trifluoromethoxy)-1H-indole **B** Schematic representation for medicinal chemistry optimization. R1 and R2 substitutions are indicated. **C** Synthesis route of CAD204520 (4-[2-[2-[3-propyl-6-(trifluoromethoxy)-1H-indol-2-yl]-1-piperidyl]ethyl]morpholine). Synthetic route (a) to (e) is depicted and described in the methods section. **D** Determination of the protein ATP hydrolysis activity in the presence of compound CAD204520 at pH 7. The figure displays ATPase activity determined by measuring the amount of liberated phosphate from ATP hydrolysis. Data is presented as a fitted curve which has been normalized to the maximal enzyme activity with subtraction of background signal from spontaneous hydrolysis of ATP. Error bars denote the mean \pm SD (standard deviation) of 3 replicates. Statistical significance (** $P \leq 0.01$; *** $P \leq 0.001$) was determined by two-way ANOVA using Bonferroni's correction for multiple comparison testing. **E** Binding sites of CAD204520 and thapsigargin. Superposition of the SERCA-CAD204520 complex with SERCA-thapsigargin (PDB ID: 2AGV). The binding sites are both in the transmembrane region, separated by transmembrane helix M3. Thapsigargin is shown as cyan surface representation. **F** Superposition of SERCA-CAD204520 with SERCA-CPA (PDB ID 3FGO), viewed roughly along the membrane normal. CAD204520 and CPA are shown as orange and green sticks, respectively. **G** Superposition of SERCA-CAD204520 with SERCA-BHQ (PDB ID 2AGV) viewed roughly along the membrane normal. CAD204520 and BHQ are shown as orange and light blue, respectively. **H** Superposition of SERCA-CAD204520 with SERCA-Cpd7 (PDB ID 5NCQ), viewed along the membrane plane. CAD204520 and Cpd7 are shown as orange and magenta sticks, respectively. **I** Binding sites of CAD204520 and thapsigargin, as seen roughly along the membrane normal. Superposition of SERCA bound to CAD204520 (light blue cartoon and orange sticks, respectively) with SERCA bound to thapsigargin (grey cartoon and sticks, respectively). Glycine257, which is mutated to valine in the thapsigargin resistant mutant, is indicated by a red sphere. In the SERCA-thapsigargin complex, Phe256 has undergone a displacement that is likely to be impaired by a valine residue in position 257.

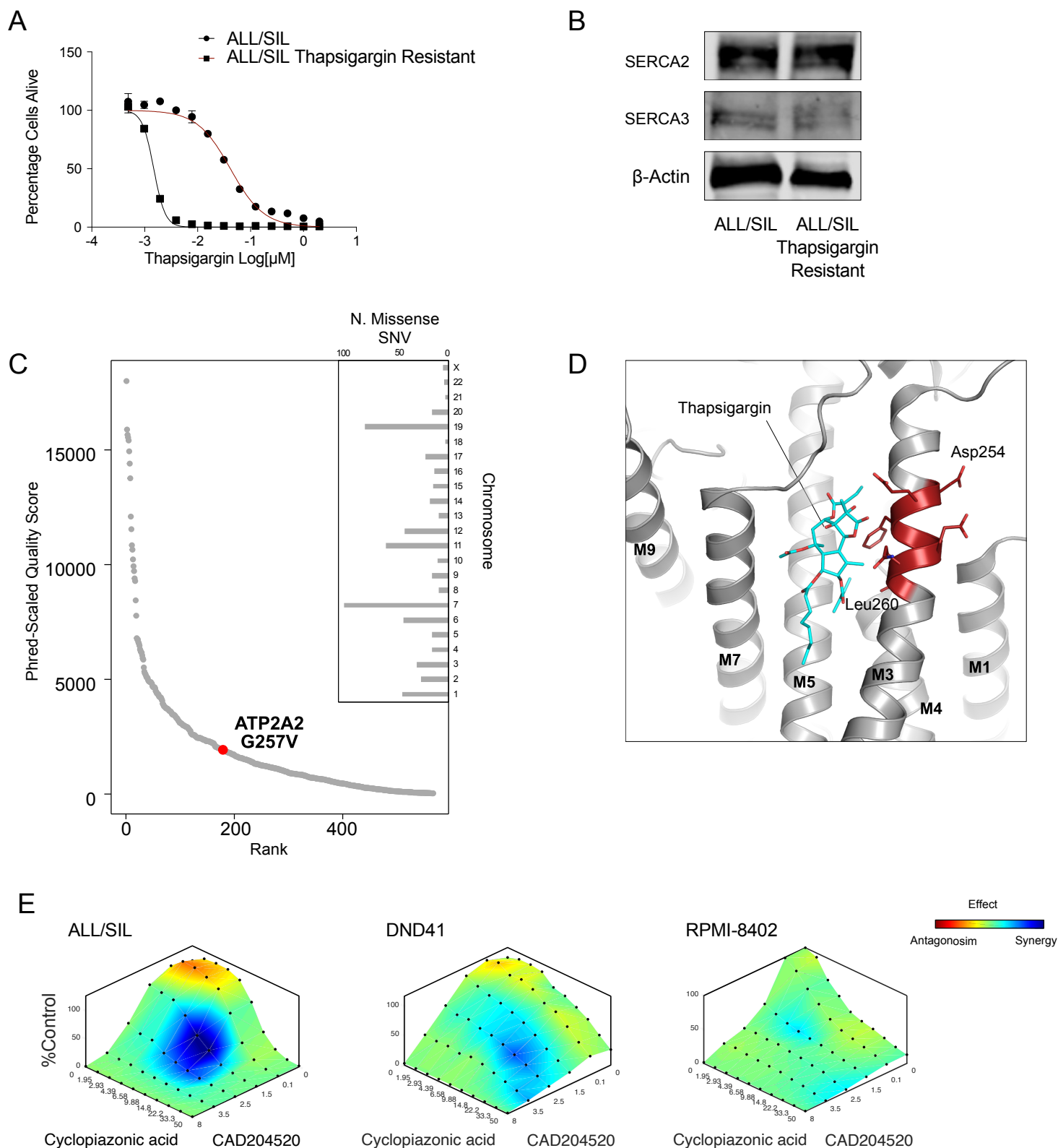


Figure S2 (Related to Figure 2): Identification of a thapsigargin resistant T-ALL cell line

A) Effects of Thapsigargin (left) and CAD204520 (right) on cell viability after 72 hours of treatments in ALL/SIL and ALL/SIL thapsigargin-resistant cell lines. Error bars denote \pm SD of a minimum of 2 replicates. **B**) Western blot showing the expression of SERCA2 and SERCA3 in naïve and resistant ALL/SIL. -actin was used as a loading control. **C**) Phred-scale analysis of exonic single nucleotide variation (SNV) occurring in the ALL/SIL thapsigargin resistant cell line. Inset shows number (N.) of variation (SNV) occurring per chromosome. **D**) Thapsigargin resistance mutation hot spot region on helix M3. Thapsigargin and SERCA residues 254-260 are shown in stick representation and colored cyan and red, respectively. **E**) Surface plots analysis of ALL/SIL, DND41 and RPMI-8402 T-ALL cells lines and a primary NOTCH1 mutated T-ALL sample treated vehicle, CAD204520, cyclopiazonic acid, or CAD204520 plus cyclopiazonic acid. Each point represents an independent measurement representative of three biological replicates. Plots were generated using Combeneft script by MATLAB R2018 and represent the Loewe (dose-effect based approach) analysis. A color scale bar represents level of drug antagonism or synergism.

A

T-ALL Cell Line	NOTCH Protein Domains		CAD204520 IC ₅₀ [μM]
	Heterodimerization (HD)	Proline, Glutamic Acid, Serine and Threonine (PEST)	
ALL/SIL	Mutated	Mutated	2.1
DND41	Mutated	Mutated	3.1
RPMI-8402	Mutated	WT	2.5
PF382	Mutated	Mutated	1.5
SKW-3/KE-37	WT	Mutated	1.5
PEER	WT	WT	9.9
Loucy	WT	WT	4.8
MOLT16	WT	WT	2.3
HSB2	WT	WT	9.5
CTV-1	WT	Mutated	0.8
REC-1	WT	Mutated	1.4
MAVER-1	WT	WT	12.7
Mino	WT	Mutated*	5.9

* PEST domain mutation p.Q2487* (exon 34). The effect of this mutation is to be considered ligand-dependent. E. Silkenstedt, F. Arenas, B. Colom-Sanmarti et al. Notch1 signaling in *NOTCH1*-mutated mantle cell lymphoma depends on *Delta-Like* ligand 4 and is a potential target for specific antibody therapy. J. Exp. Clin. Cancer Res, 2019. **38**(1), 446.

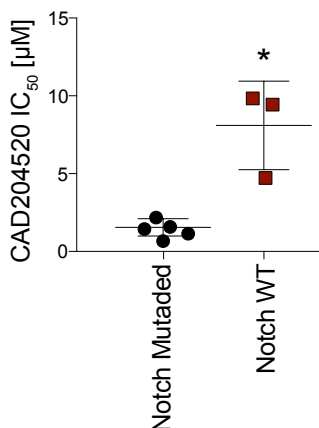
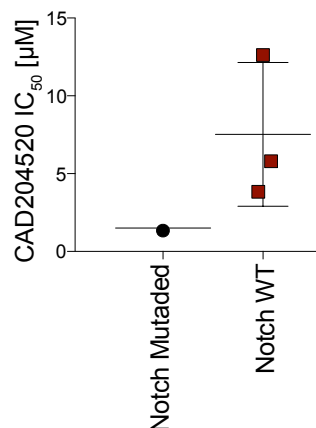
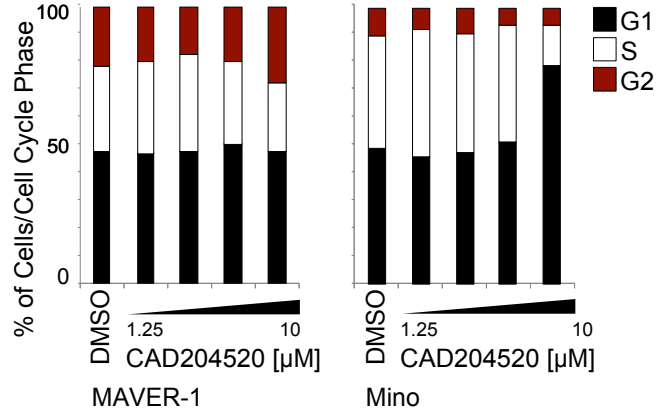
B**C****D**

Figure S3 (Related to Figure 3): Effect of CAD204520 on NOTCH1 wild type T-ALL and MCL lines

A) Table representing NOTCH1 mutational status in T-ALL and MCL lines. **B)** Scatter dot plot representing IC₅₀ [μM] of CAD204520 in NOTCH1 mutated (n=5) or NOTCH1 WT (n=3) T-ALL or in NOTCH1 mutated (n=1) or NOTCH1 WT (n=3) MCL (shown in **C**) cell line. Statistical significance (*P ≤ 0.05) was determined by a non-parametric t-test (Mann-Whitney). **D)** Effect of CAD204520 treatments on cycling MAVER-1 and MINO cells. Percentage of DNA content following four days of treatment with the indicated concentrations of CAD204520 on each cell cycle phase is indicated. A minimum of 20,000 events was collected for each condition.

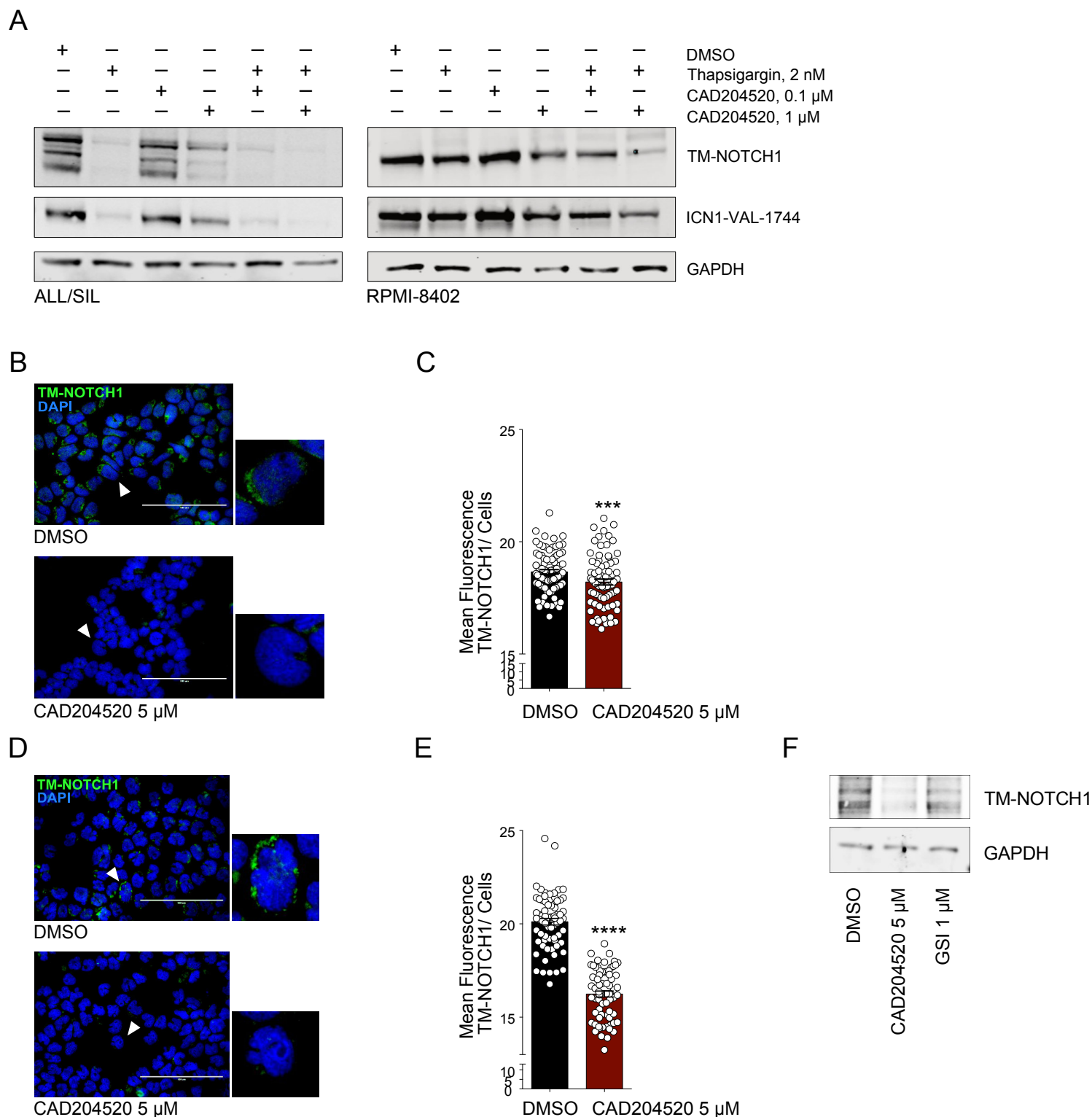


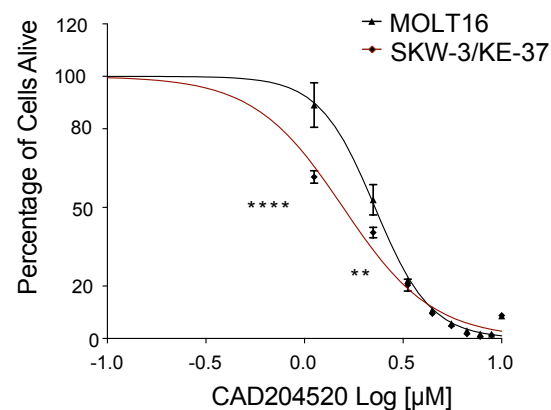
Figure S4 (Related to Figure 4): Effects of CAD204520 on NOTCH1 trafficking

A) Effect of CAD204520 and thapsigargin treatment for 24 hours on NOTCH1 (N1) processing and activation in T-ALL cell lines. The immunoblot was incubated with an antibody against the C-terminus of NOTCH1 that recognizes the transmembrane subunit (TM) and an antibody that recognizes the cleaved NOTCH1 (ICN1). GAPDH was used as a loading control. **B)** Effect of 24 hours of CAD204520 on NOTCH1 cell surface staining as assessed by immunofluorescence in DND41 T-ALL cells. Scale bars: 100 μ m. **C)** Quantitative immunofluorescence analysis of NOTCH1 surface signal in DND41 cells after 24 hours of CAD204520 treatment. Error bars denote the mean \pm standard deviation (SD) of fluorescence of 70 single cells/nuclei (arbitrary units); Statistical significance among groups was determined by unpaired t-test ($***P \leq 0.001$). **D)** Effect of 24 hours of CAD204520 on NOTCH1 cell surface staining as assessed by immunofluorescence in REC-1 T-ALL cells. Scale bars: 100 μ m. **E)** Quantitative immunofluorescence analysis of NOTCH1 surface signal in REC-1 cells after 24 hours of CAD204520 treatment. Error bars denote the mean \pm standard deviation (SD) of fluorescence of 70 single cells/nuclei (arbitrary units); Statistical significance among groups was determined by unpaired t-test ($***P \leq 0.001$). **F)** Effect of 24 hours treatment of CAD204520 on NOTCH1 (N1) processing and activation in REC-1 cell line. The blot was incubated with an antibody against the C-terminus of NOTCH1 that recognizes the NOTCH1 transmembrane subunit (TM). GAPDH was used as a loading control.

A

CELL LINES	GENOMIC PROFILE
MOLT16	<i>SIL-TAL1</i> t(3;11)(p21;p13)/ <i>LMO2</i> translocation with non- <i>TR@</i> partner t(8;14)(q24;q32)/ <i>TRAD@-MYC</i> <i>CDKN2AB</i> biallelic deletion <i>PTEN</i> c.735-736insCTTA p.P246fs*12 (exon 7)
SKW-3/KE-37	t(8;11)(p11-12;p13)/ <i>LMO2</i> translocation with non- <i>TR@</i> partner t(8;14)(q24;q32)/ <i>TRAD@-MYC</i> <i>CDKN2AB</i> biallelic deletion <i>PTEN</i> biallelic deletion <i>NOTCH1</i> c7375C>T p.Q2459* (exon 34)

B



C

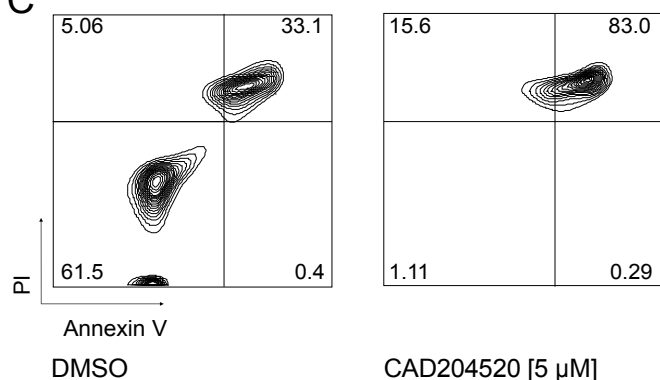


Figure S5 (Related to Figure 5): Genomic characterization of MOLT16 and SKW-3/KE-37 T-ALL cell lines

A) Genomic characterization of MOLT16 and SKW-3/KE-37 T-ALL cell line. The karyotype and the mutational analysis are indicated. **B)** Effects of CAD204520 on cell viability after 72 hours of treatments in MOLT16 and SKW/KE-37 T-ALL cell lines. Error bars denote \pm SD of 2 replicates. Statistical significance among groups was determined by 2-way ANOVA (** $P \leq 0.01$, *** $P \leq 0.001$). **C)** Pro-apoptotic effect of CAD204520 treatment. Annexin V/propidium iodide staining of primary NOTCH1 mutated T-ALL cells following 72 hours of treatment with the indicated concentrations of CAD204520. A minimum of 20,000 events was collected for each condition.

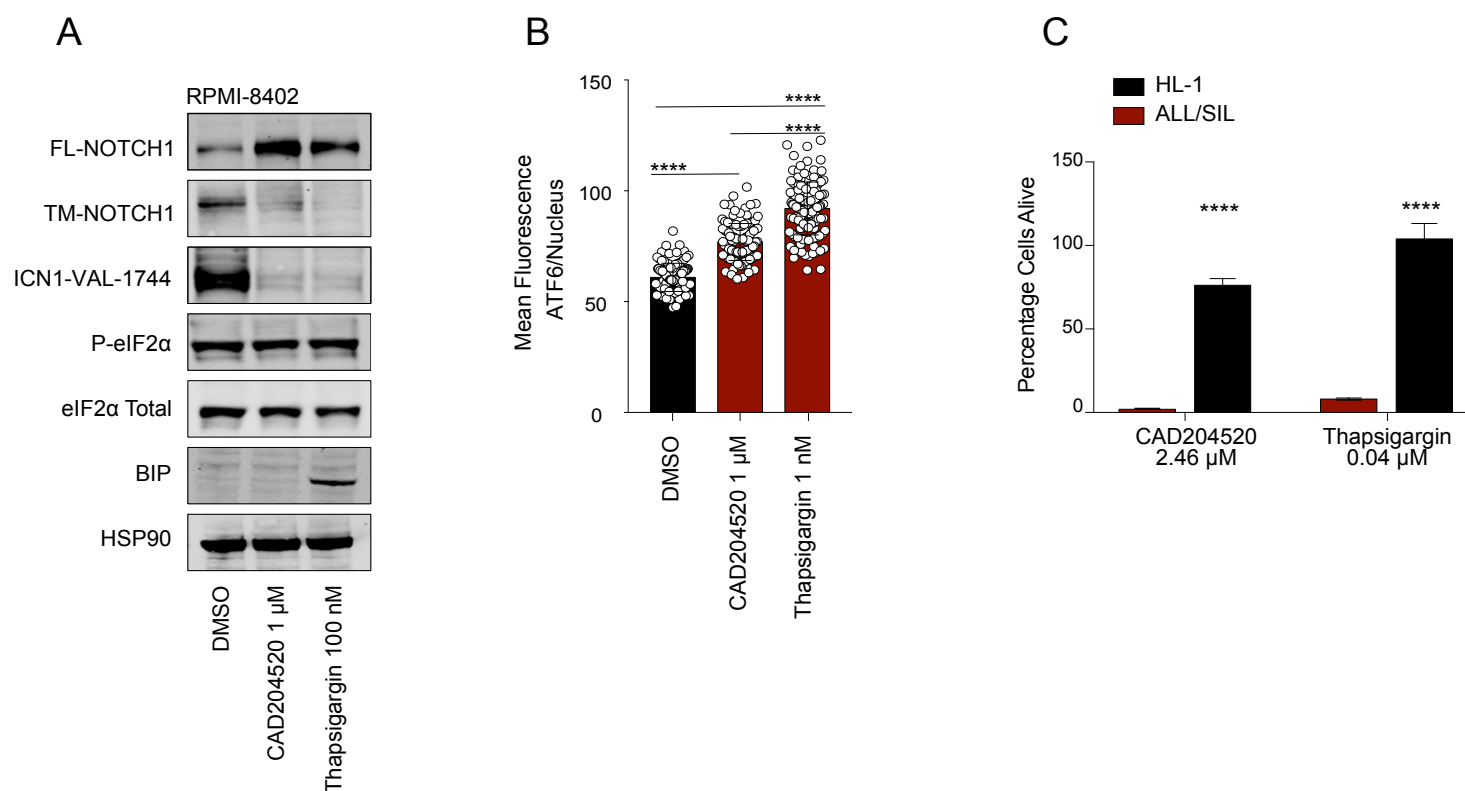


Figure S6 (Related to Figure 6): Consequences of CAD204520 on UPR pathway

A) Effect of CAD204520 and thapsigargin treatment for 24 hours in RPMI-8402 cell lines. The immunoblot was stained with an antibody against the C-terminus of NOTCH1 that recognizes the furin-processed NOTCH1 transmembrane subunit (TM) and the unprocessed NOTCH1 precursor (FL), an antibody that recognizes the cleaved NOTCH1 (ICN1), and antibodies that recognize the activation of the UPR pathway. P-eIF2, eIF2, total BiP. HSP90 was used as a loading control. **B)** Quantitative immunofluorescence analysis of nuclear ATF6 signal in ALL/SIL cells after 24 hours of CAD204520 treatment. Error bars denote the mean \pm standard deviation (SD) of fluorescence of 70 single cells/nuclei (arbitrary units); Statistical significance among groups was determined using one-way ANOVA with Bonferroni's correction for multiple comparison testing ($***P \leq 0.001$). **C)** Effect of CAD204520 and thapsigargin treatment in HL-1 and ALL/SIL cell lines. Histograms show percentage of cell alive after 72 hours of treatment at indicates doses. Error bar denotes the mean \pm SD of a minimum of three replicates. Statistical significance among groups ($****P \leq 0.0001$) was determined by 2-way ANOVA.

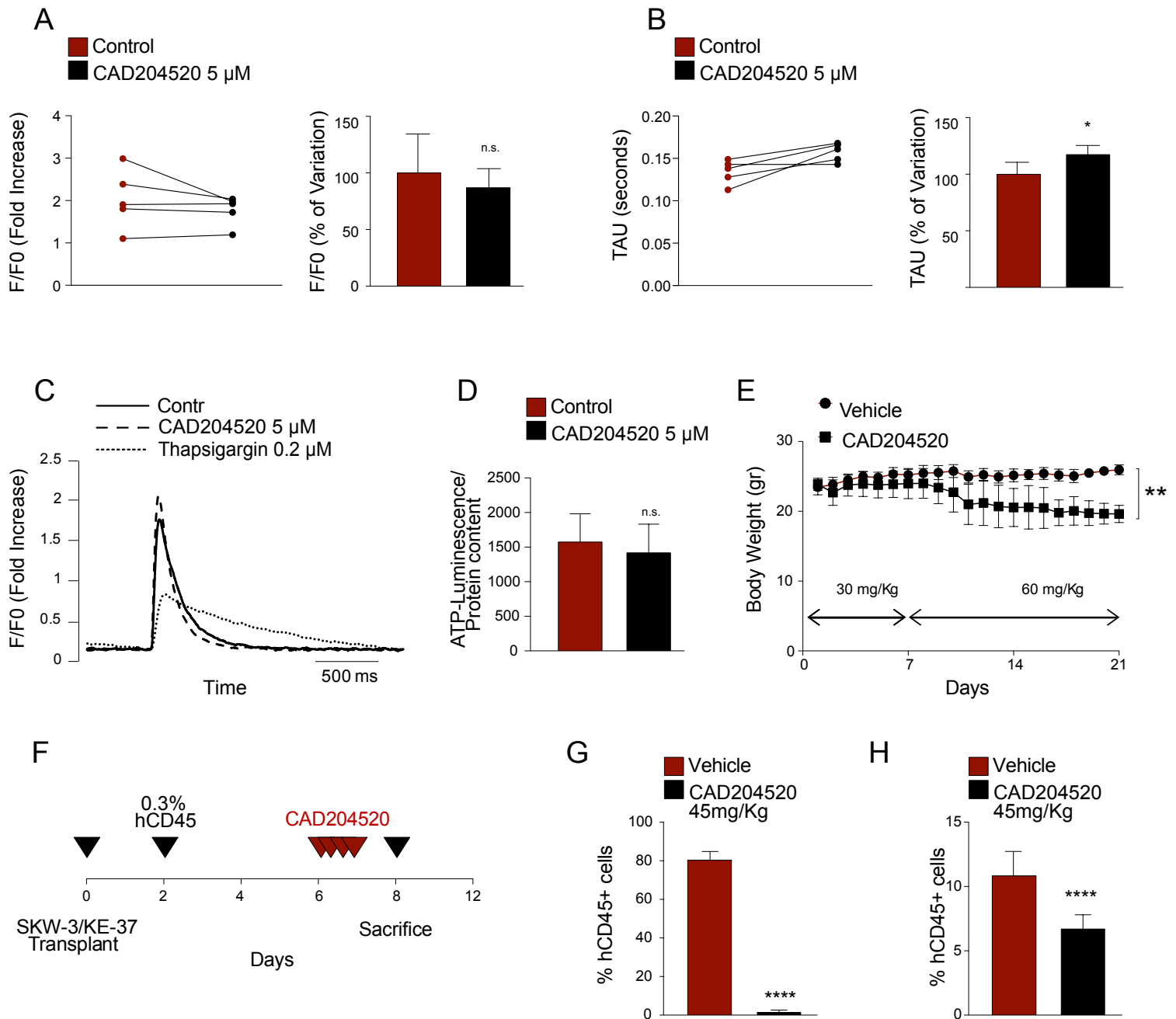


Figure S7 (Related to Figure 7): Preclinical toxicity and activity of CAD204520

A-B left panels): Effect of CAD204520 treatment on rat cardiomyocyte calcium transients. Single experiments are represented by two dots interconnected by a solid line. Specifically, the line between dots connects the quantification of the amplitude of the calcium transient (A: F/F0) and the time required for cytosolic calcium removal (B: TAU), before and after the CAD204520 (5 μ M) treatment compared to control (Control). **A-B right panels):** Mean percentage variation of CAD204520 treated cardiomyocytes on the same parameters. Graph bars: mean \pm SD of the 5 CAD204520 treated cardiomyocyte groups. Statistical significance comparing CAD204520 treated cells vs. Control cells (* P < 0.05) was determined by a non-parametric t-test (Mann-Whitney). **C)** Representative examples of calcium transients (normalized traces: fold increase) recorded from control (solid line), CAD204520 (5 μ M) (dashed line), and thapsigargin (0.2 μ M) (dotted line) ventricular myocytes. **D)** ATP Luminescence induction normalized to cellular protein content in rat cardiomyocytes treated either with CAD204520 or vehicle (Control). Error bars denote the mean \pm SD of 6 replicates. Statistical significance comparing CAD204520 treated cells vs. vehicle treated cells (not significant = n.s.) was determined by a non-parametric t-test (Mann-Whitney). **E)** Effect of administration of CAD204520 on body weight. Error bars denote the mean \pm SD of 6 replicates. Animals were treated for 6 days with 30mg/Kg of CAD204520 and subsequently treated with 60 mg/Kg daily. Statistical significance (** P \leq 0.01) was determined by a 2-way ANOVA analysis. **F)** Representative schema of CAD204520 in vivo studies. **G)** Effect of CAD204520 on T-ALL leukemia burden in a SKW-3/KE-37 xenografted murine model. Anti-leukemic activity of CAD204520 assessed by measuring hCD45+ cells after 4 days of CAD204520 treatment (45 mg/kg/OS BID) or vehicle (tween-80 0.5% w/v and HPMC 1.0% w/v). Error bars denote the mean \pm SD of 9 CAD204520 treated animals or the mean \pm SD of 8 replicates vehicles treated mice. Statistical significance for treated vs. vehicle (**** P \leq 0.0001) was determined by non-parametric t-test (Mann-Whitney). **H)** Antileukemic activity of CAD204520 in hCD45+ spleen infiltrating cells in a SKW-3/KE-37 xenografted murine model after 5 days of CAD204520 treatment (45 mg/kg/OS BID) or vehicle (tween-80 0.5% w/v and HPMC 1.0% w/v). The number of hCD45+ cells per field were represented as percentage relative to vehicle control. Error bars denote the mean \pm SD of 13 fields from 2 mice treated with CAD204520 or the mean \pm SD of 12 fields from 3 vehicle treated mice. Statistical significance for treated vs. vehicle (**** P \leq 0.0001) was determined by non-parametric t-test (Mann-Whitney).

Compound	Structure	ATP hydrolysis IC ₅₀ [μM]			SERCA Ligand Efficiency Index (LE)
		H ⁺ -ATPase	Na ⁺ ,K ⁺ -ATPase	Ca ²⁺ -ATPase (SERCA)	
CAD204522		126.80 ±14.94	22.56 ±5.48	30.14 ±5.61	0.20
CAD307496		37.68 ±6.22	20.09 ±2.76	58.86 ±6.50	0.21
CAD204521		21.81 ±4.50	0.28 ±0.07	7.75 ±1.68	0.26
CAD204630		>333	0.04 ±0.01	1.56 ±0.45	0.25
CAD204631		71.25 ±18.00	3.16 ±0.41	18.00 ±3.25	0.21
CAD305666		9.74 ±1.67	0.39 ±0.20	1.03 ±0.22	0.27
CAD204519		84.22 ±24.37	0.01 ±0.02	0.55 ±0.12	0.28
CAD204520		26.90 ±2.98	8.30 ±0.95	0.34 ±0.03	0.29
CAD306749		7.75 ±2.69	0.59 ±0.17	0.32 ±0.19	0.22
CAD306750		16.80 ±2.68	1.21 ±0.37	2.62 ±0.83	0.22

Table S1 (Related to Figure 1 and Figure S1): H⁺, Na/K⁺, Ca²⁺ ATP hydrolysis of 2-(2-pyridyl)-6-(trifluoromethoxy)-1H-indole derivatives

Data of compounds fitted to the pharmacophore model. ATPase hydrolysis activity toward H⁺, Ca²⁺ and Na⁺/K⁺-ATPase is indicated as the half maximal inhibitory concentration (IC₅₀) and expressed in μM. The ligand efficiency index is equal to LE = 1.4(-logIC₅₀)/N. N is the number of non-hydrogen atoms.