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# *CAD204520 targets NOTCH1 PEST domain mutations in lymphoproliferative disorders*

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# 1. INTRODUCTION

## 1.1 NOTCH background and protein structure

In the first decades of 1900s, John S. Dexter and later T.H. Morgan observed mutant flies with "beaded" wings in *Drosophila melanogaster*[1, 2]. Subsequent studies demonstrated that this was caused by a heterozygous deletion of a gene located on X chromosome of *Drosophila*[3]. This unusual phenotype provided the gene name that we currently know: "*NOTCH"*.

Only in 1980s, in the same model, *NOTCH* was isolated, cloned and sequenced for the first time, leading to the discovery of its role and structure[4-6].

Currently we know that in mammalian genomes, *NOTCH* encodes for four paralogs: NOTCH1, NOTCH2, NOTCH3 and NOTCH4[7, 8]. These proteins are single-pass type I transmembrane receptors deputed to receive signals from transmembrane ligands expressed on neighboring cells and play the role to transduce the extracellular signals into the cells[9]. These proteins are synthesized as single precursors (proNOTCH) that will then undergo to a series of cleavages, resulting in the release of NOTCH active form. The activated subunit works like a transcription factor, translocating into the nucleus and activating its target genes.

The Notch pathway starts with a first cleavage by a furin-convertase protease (S1) in the *trans*-Golgi network. This cleavage results in the release of a heterodimer receptor consisting in an extracellular domain (NECD) that interact in a non-covalently way, via a heterodimerization domain (HD), to a transmembrane (NTM) and intracellular subunit (NICD)[8].

The extracellular subunit contains several epidermal growth factor-like (EGF-like) motif repeats involved in ligand-receptor interaction. Specifically, NOTCH1 and NOTCH2 contain 36 EGF-like repeats, meanwhile NOTCH3 and NOTCH4 contain 34 and 29 repeats, respectively[10, 11]. These are followed by three copies of LIN12-Notch repeats (LNRs) sequence motif[12] which stabilize the HD domain by holding the two NOTCH subunits together. LNRs and HD domain, together, work like a negative regulatory region (NRR) preventing a ligand-independent activation of the Notch signaling pathway[13].

The extracellular domain is linked to the C-terminal intracellular domain by a small transmembrane domain (TM) and, it is characterized by a RAM (RBPJ-associated module) domain, linked through a nuclear localizing sequence (NLS), to a series of ankyrin repeats (ANK). In NOTCH1, NOTCH2 and NOTCH3, this is followed by a NOTCH cytokine response (NCR) region, absent in NOTCH4[14]. Finally, all paralogs carry a transactivation domain (TAD), required for activating the transcription, and lastly a region rich in proline (P), glutamate (E), serine (S) and threonine (T) (PEST), which is involved in NOTCH degradation control[15] (Figure 1A).

### 1.2 Notch pathway activation

In mammalian, the ligands necessary for the activation of the canonical Notch signaling belong to Delta (Delta-like 1, 3 and 4) and Jagged (Jagged 1 and 2) ligand families. They are expressed on cells surface and have redundant and unique functions[16]. These ligands are single-pass transmembrane proteins, exactly like NOTCH receptors. They are characterized by multiple EGF-like repeats and cysteine-rich sequences known as the Delta-Serrate-Lag2 (DSL) motif, necessary for the interaction with NOTCH receptors of neighbor cells[17].

After the ligand-receptor binding, NOTCH receptors undergo to a conformational change that leads to two subsequent proteolytic cleavages: the first one (S2) mediated by a metalloprotease ADAM10/17 (A Disintegrin And Metalloprotease 10/17). Its activity causes a conformational change in the LNR–HD complex, which results in the proteolytic cleavage of the transmembrane-intracellular domain of the receptor. The following cleavage (S3) is completed by the γ-secretase, a complex composed by four proteins: presenilin (PS), nicastrin (NCT), Pen2, and Aph1[18, 19]. This step results in the release of the NICD from the plasma membrane.

NICD is considered the "active form" of NOTCH protein, in fact it translocates into the nucleus, where, in combination with a transcriptional complex composed of CSL (CBF-1/suppressor of hairless/LAG-1, also known as RBP-Jk), mastermind-like (MAML1–3) coactivator, and other proteins[20, 21], it regulates the transcription of several target genes[22] (Figure 1B).

Nowadays, we know that Notch pathway plays an important role in several processes such as stem cell maintenance, cell differentiation and tissue homeostasis[16, 23]. Consequently, given its involvement in all these important processes, is also clear that a dysregulation in Notch pathway is involved in several diseases[24-27].

### 1.3 Notch aberration in tumorigenesis

Notch is a highly conserved signaling pathway[16] and its dysregulation or loss of function is associated to multiple diseases: from developmental syndromes to adult onset diseases[15, 24] and several types of cancer[25].

In oncological field, the particularity of Notch signaling is that it can play either an oncogenic or tumor-suppressor role depending on the cellular context[28, 29]. Several evidences showed that Notch-activating mutation can be involved in

different types of cancer, such as in breast cancer, colorectal cancer, non–small cell lung carcinoma (NSCLC)[27], T-cell acute lymphoblastic leukemia (T-ALL), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL) and others[30-34]. In the same time, *NOTCH* inactivating mutations can still lead to tumor development. This evidence has been confirmed in the skin, head and neck squamous cell carcinoma (HNSCC) and in hepatocellular carcinoma (HCC)[35-38]. Hence, the abnormal regulation of the Notch pathway may occur by a variety of mechanisms including mutational activation or inactivation, overexpression, but also post-translational modifications and epigenetic regulation[39, 40].

The role of mutated Notch pathway in so many different diseases has led to increasing knowledge on its origin and involvement in diseases. Despite this, especially for cancer, its targeting did not still show very promising results.

Therefore, the goal of this project is to target one the most common activating *NOTCH* mutation in lymphoproliferative diseases: PEST domain mutations, which presence lead to increased cells proliferation and resistance to apoptosis [41].

### 1.4 *NOTCH* mutation in hematological diseases

The first evidence of Notch signaling involvement in hematological malignancies was highlighted in 1991, when Ellisen and colleagues described for the first time the oncogenic potential of *NOTCH1* in T-ALL. They identified a rare translocation, t(7;9)(q34;q34.4), that juxtaposes the promoter elements of the T-cell receptor-β (*TCRβ*) to the 3' end portion of the *NOTCH1* gene leading to an overexpression of the NOTCH1 active form[42].

Currently, we know that activating *NOTCH1* mutations in the juxtamembrane HD domain are the main hotspot site for class 1 and class 2 nucleotide

variations. This lead to an enhanced instability of NOTCH1 and a constitutively activation of the Notch1 pathway targets[43, 44]. These type of mutations occur in more than 50% of T-ALL[45].

A second hotspot region for mutations is in the *NOTCH1* exon 34 of C-terminal PEST sequence. PEST domain represents the ubiquitination site for the E3-ubiquitin ligase and it is necessary for maintaining the stability and turnover of the intracellular NOTCH1 domain[22]. Short insertions or deletions in this hotspot disrupt the binding site of the E3-ubiquitin ligase, reducing the cleavage and impeding the inactivation of the intracellular domain[43].

C-terminal PEST domain mutations are observed in 5-20% of cases of CLL, MCL[46, 47] and in less than 5% in diffuse large B-cell (DBCL) and follicular lymphoma (FL)[48, 49]. In these diseases, the most frequent mutation is a 2-bp deletion in exon 34 that generates a premature stop codon (P2514fs*4) leading to a truncation of the C-terminal PEST region[50]. Since there is the lack of its degradation, these mutations cause an over-activation of Notch1 signaling.

While the role of *NOTCH1* mutations in prognosis has not been established for several tumors and, for others, it remains completely unclear[51-54], PEST mutations identify a subset of lymphoproliferative disorders characterized by a poor prognosis[51-55].

### 1.5 The role of *NOTCH1* mutation in chronic lymphocytic leukemia and mantle cell lymphoma

Chronic lymphocytic leukemia is the most common leukemia of the western world and currently is still an incurable disease[56]. The first evidence of Notch pathway involvement in CLL was related to *NOTCH2*, whose mutation led to increased cell survival[57]. Later, it was realized that also *NOTCH1* plays a key role in CLL. For example, *Rosati et al.* demonstrated that in CLL there was a

constitutively expression of NOTCH1 and of its ligands, leading to an apoptosis cells resistance[58]. Subsequent studies identified the presence of *NOTCH1* mutations as often related to advanced disease, and to an increased risk of Richter's transformation, especially when it is associate with trisomy of chromosome 12 (+12)[59-61]. Moreover, studies reported that Notch1 signaling may create conditions that favor the development of drug resistance, emphasizing the importance of its targeting[55]. In fact, a study where CLL cells were cocultured with human BM-mesenchymal stromal cells (hBM-MSCs) demonstrated a resistance to apoptosis both spontaneously and following induction with various drugs such as fludarabine, cyclophosphamide, bendamustine, prednisone and hydrocortisone. This was reversed only with the addition of anti-NOTCH1 antibodies or γ-secretase inhibitors[62]. Another study associated the mutation to immunotherapy resistance, exactly to the anti-CD20 monoclonal antibody (mAb) rituximab. In fact, it was demonstrated that the use of rituximab, in association with fludarabine and cyclophosphamide (FCR protocol) in patients with *NOTCH1* mutations, doesn't present benefits. The addition of anti-CD20 mAb, in presence of *NOTCH1* mutation, doesn't increase the clinical response or improve the overall survival[63].

Similarly to CLL, the presence of *NOTCH1* mutation is a negative prognostic factor in MCL. MCL is an incurable subtype of B-cell non-Hodgkin lymphomas (NHLs) characterized by a heterogeneous clinical course[64, 65]. *NOTCH1* PEST domain mutation in MCL is associated with a poor prognosis and with shorter survival rates compared to *NOTCH1* wild-type (WT) MCL. In fact, the median overall survival (mOS) is 1.4 years compared with 3.8 years for *NOTCH1* WT cases[51, 66-68].

All these data position NOTCH1 as a potential molecular target for cancer treatment and suggest the need to find novel treatment approaches for impairing Notch1 dysregulated-signaling in these diseases.

### 1.6 Targeting Notch trafficking

Despite several therapeutic efforts, currently there are no specific treatment for diseases with aberrant Notch signaling.
In recent years, research has focused on antibody-based strategies or small molecules approaches. An example is characterized by the development of new antibodies able to block the interaction between NOTCH ligand and its receptors or monoclonal antibodies against clinically relevant mutant receptors[69, 70].
Another targeting-approach is depicted by modulators of the γ-secretase complex (GSI), which is required for the release of the active subunit of NOTCH1. Different studies showed that GSI treatment induces G0/G1 arrest in *NOTCH1* mutated cells, with rapid clearance of intracellular NOTCH1[71].

Unfortunately, GSIs demonstrated to be poorly tolerated for their on-target gastro-intestinal toxicity caused by a lack of substrate specificity that results in the combined inhibition of mutated and WT NOTCH1 and NOTCH2 of intestinal progenitor cells[72]. Based on the anti-NOTCH effects, others GSIs have been formulated trying to reduce the on-target effects, without effective results. For example, some GSIs were coupled with other drugs or molecules, such as with glucocorticoids[73]. This approach led to the lack of gastrointestinal toxic effects but also to an increased risk of developing skin cancers since Notch1 signaling has the role of onco-suppressor in this tissue[36]. Despite their potential, the absence of selectivity and the imponent side effects, limited their clinical transition for *NOTCH1* mutated malignancies[74].

Different is the case of small molecules targeting the sarco-endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA). SERCA proteins belongs to a group of evolutionarily conserved proteins of the P-type ATPase family[75]. It is important

for maintaining intracellular $Ca^{2+}$ homeostasis through the $Ca^{2+}$ pumping from cytosol to ER[76] and its altered activity is associated with cardiovascular[77], neurological[78], renal[79] and metabolic diseases[80].

Recently, it has been shown that specific SERCA inhibition blocks Notch trafficking and its activation, emerging as a druggable site for oncological disease characterized by *NOTCH* mutation[81]. In 2013, our group and Dr. Stegmaier's laboratory identified SERCA as a potential therapeutic target selectively in *NOTCH1* mutated cancers, overcoming the innate limitations associated with other small molecules such as the GSIs[82] that equally target WT and mutated NOTCH1 proteins[82, 83].

Specifically, from a gene expression-based high-throughput small molecules (GE-HTS) and a cDNA overexpression screening using cell-based assays that reporting *NOTCH* transcriptional activity, the team discovered new targets that preferentially impair *NOTCH1* mutations[82, 84]. From this screening, one of the top compound hits was thapsigargin, a guaianolide extracted from the *Thapsia garganica* plant, which is a noncompetitive inhibitor of SERCA. In parallel, among the top cDNA hits there was *ATP2A2*, gene, which encodes for SERCA2[82].
Thapsigargin, once bound to SERCA, showed an impairment in NOTCH1 trafficking, limiting the access of pre-processed NOTCH polypeptides to the γ-secretase complex. This effect was more impactful in *NOTCH*1 mutated cancers[83], highly dependent from its signal[85].
At the same time, SERCA confirmed to have a fundamental role in this pathway, because its inhibition impairs the trafficking of mutated NOTCH1 receptors and induces a proliferation arrest in *NOTCH1*-mutated cells[86].

Thapsigargin showed an on-target activity in mouse models of human T-ALL and interfered with Notch signaling in *Drosophila*[87, 88]. However, due to the

rapid disruption of $Ca^{2+}$ homeostasis, thapsigargin induced cardiac toxicity, limiting its application in a clinical setting[81].
Hence, although its selectivity provided a therapeutic window never observed before with other treatment approaches, there remained necessary to develop new SERCA inhibitor characterized by lower off-target effects.

## 1.7 CAD204520 as a new therapeutic strategy for *NOTCH1* mutated cancer

Continuous efforts in the field have led our group to identify a new SERCA inhibitor, CAD204520, that showed *in vitro* and *in vivo,* an anti-*NOTCH1* activity without the off-target toxicity previously observed with thapsigargin[81].

It was identified from a library screening of 191'000 compounds, investigated to discover new potent P-type ATPase inhibitors[89]. The resulted compounds were subsequently counter-screened to identify molecules that preferentially inhibit $Ca^{2+}$-ATPases and, CAD204520 showed to be the best match for SERCA inhibition, reducing its ATP-hydrolysis activity[81]. CAD204520 was tested in a panel of T-ALL and MCL cell lines with activating mutations in the HD domain of *NOTCH1* and/or deletions in the PEST domain, showing an inhibition of leukemia growth after treatment. In addition, *NOTCH1* mutated cell lines showed to be more sensitive to CAD204520 compared to *NOTCH1* WT tumor cells[81], in line with the effect showed by thapsigargin. In addition, CAD204520 resulted in an effective treatment in a *NOTCH1* mutated T-ALL also in an *in vivo* model, without causing overt $Ca^{2+}$-related cardiac toxicity.

Based on the promising result of CAD240520 in a *NOTCH1* mutated leukemia model, the main goal of my work is to extend the experience in the field by providing the context for targeting Notch1 pathway through SERCA inhibition in tumors with isolated PEST domain mutations.

# 2. AIMS OF THE STUDY

*NOTCH1* gene mutations in MCL and CLL have been described in about 5–20% of cases and are associated with significantly shorter survival rates, identifying a subset of high-risk patients with a worse prognosis.

Our preliminary studies demonstrated that CAD204520 impairs *NOTCH1* mutated cells growth through the suppression of SERCA without relevant side effects. For this reason, my project seeks to apply approaches to validate the use of CAD204520 as a new molecule for the treatment of *NOTCH1* PEST-mutated lymphoproliferative diseases.

Given the high frequency of CLL disease, we collected CLL patient samples obtained under their consent. The primary samples were first characterized to define the stage of the disease and especially the *NOTCH1* mutation status at the date of collection. CAD204520 has been tested to observe whether it inhibits leukemia growth using multiple approaches including western immunoblotting, ATP-based and flow cytometry assay.

The results allowed us to compare the response in *NOTCH1* mutated and WT samples. Further comparison experiments were conducted using several commercial *NOTCH1* mutated cell lines to simultaneously evaluate the response in different models.

Finally, we developed a preclinical *NOTCH1* PEST mutated murine model in order to confirm *in vivo* the efficacy and safety of CAD204520 in lymphoproliferative PEST-mutated disorders.

CLL and MCL are still incurable with the current available therapies and the presence of *NOTCH1* mutations represents an additional obstacle to the

possible success of the treatment. Therefore, we also evaluated if the addition of CAD204520 to the current available target therapies could increase the response to treatment.

Thus, all our efforts validated CAD204520 as a new therapeutic strategy in *NOTCH1* PEST mutated lymphoproliferative disorders increasing their chance treatment response and improving the current unfavorable disease outcome.

# 3. MATERIALS AND METHODS

## 3.1 Cell Lines

The human cell lines ALL-SIL, SKW-3/KE-37, CTV-1, MEC-1, JEKO-1, REC-1, and Granta-519 were purchased from the Leibniz Institut DSMZ-German collection of microorganism and cell cultures (Germany). Cells were cultured in RPMI 1640 (#MT10040CV, Thermo Fisher Scientific, Waltham MA, USA) with 10% or 20% fetal bovine serum (FBS) (#10270–106, Thermo Fisher Scientific), 1% penicillin-streptomycin (P/S) (#3MT30002CI, Thermo Fisher Scientific), and 1% of MEM Non-Essential Amino Acids Solution (100X) (#11140050, Thermo Fisher Scientific), 2 mM L-Glutamine (#25030-081, Thermo Fisher Scientific) and 1% HEPES Buffer 1M (#MS013D1006, Biowest). Granta-519 were maintained in DMEM (#11960-044, Thermo Fisher Scientific) with 20% FBS, 1% P/S, and 2 mM L-Glutamine (#25030-081, Thermo Fisher Scientific). Cell lines were grown in a humidified incubator at 37 °C and 5% CO2 and monitored for mycoplasma contamination.

## 3.2 Primary Samples collection

Chronic lymphocytic leukemia (CLL) cells derived from peripheral blood (PB) were obtained from patients with CLL under an approved protocol from the Department of Medicine and Surgery at Parma University Hospital (n.29785/13/07/2021), according to the declaration of Helsinki guidelines for the protection of human rights. Lymphocytes from PB samples were isolated through a density gradient centrifugation using Lympholyte Cell Separation

Media (#CL-5020, EuroClone SpA, Italy) and cultured in IMDM (#12440-053, Thermo Fisher Scientific) with 20% FBS, and 1% P/S.

### 3.3 Karyotype Analysis and Fluorescence in Situ Hybridization

Primary peripherical blood samples were cultured for 72 hours in RPMI 1640 with 20% FBS, 1% P/S and ChromoLympho-B Proliferation MIX (with CpG-oligonucleotide DSP30 plus IL-2) (#EKAMP010M, Euroclone), to increase the leukemic B lymphocyte proliferation and improve the mitotic rate. Cell media was supplemented with 0.1 µg/mL of colcemid (#15212012, Thermo Fisher Scientific,) for 2 hours, followed by incubation in a hypotonic solution (0.075M KCl). Cells were fixed in a 3:1 methanol (#322415, Sigma-Aldrich, St. Louis, MO, USA) and acetic acid glacial fixative solution (#A6283, Sigma-Aldrich) and spread on top of Superfrost Plus microscope slides (#10149870, Thermo Fisher Scientific). For the karyotype analysis, chromosome banding was performed by quinacrine (Q-banding) staining. A minimum of 20 metaphases per sample were acquired using a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY, USA) and analyzed using NIS element software (Nikon Instruments, Inc.). For the fluorescence *in situ* hybridization (FISH) analysis, 10 µL of XL ATM/TP53 (#D-5046-100-OG, Metasystems) or set probe" XL DLEU/LAMP/12cen (#D-5055-100-TC, Metasystems) were incubated at 37 C for 12-16 hours after a phase of DNA dehydration with ethanol-scale incubation (75%-85%-100%) and DNA denaturation (75°C, 5 minutes). Slides were washed once with 0.4x saline sodium-citrate/0.3% NP40 buffer at 73° C, followed by 4 x SSC/0,1% NP-40 at ambient temperature. DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; #10236276001, Sigma-Aldrich) before microscope analysis (Eclipse 80i microscope, Nikon Instruments, Inc.). Two-hundred interphase nuclei were analyzed for each patient and DNA abnormalities were defined starting from a 5% cutoff for each

probe. For the detection of del(17p), a cutoff of 20% interphase nuclei was adopted, in line with previous findings[90].

### 3.4 Next-Generation Sequencing

DNA was extracted using a Maxwell® 16 DNA purification kit (#AS1010, Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. The concentration and purity of the DNA samples were determined with a Qubit 4 fluorometer (#33226, Thermo Fisher Scientific). Primary samples were sequenced using the Sophia Lymphoma Solution™ kit (#CS.2205.0103-00, Sophia Genetics SA, Saint Sulpice, Switzerland). Library preparation and sequencing were performed on a MySeq system (Illumina) following the manufacturer's instructions. Data were analyzed with Sophia DDM® software version 5.10.11.1 (Sophia Genetics SA). A cutoff VAF ≥10% for *TP53* gene mutations was adopted, in line with the European Research Initiative on CLL (ERIC) recommendations[91].

### 3.5 Western Immunoblot and Antibodies

Whole cell protein lysates were extracted using 1X Cell Lysis buffer (#9803S, Cell Signaling Technology, MA, USA) with Protease/phosphatase Inhibitor Cocktail 100X (#58725, Cell Signaling Technology, MA, USA). Cells were lysed on ice for 30 min with gentle stirring and centrifuged at 14,000 RPM for 10 min at 4°C. Proteins lysates were quantified using Bio-Rad Protein Assay Dye Reagent (#5000006, Bio-Rad Laboratories, Hercules, CA, USA) and the total lysate/sample was loaded for SDS-PAGE analysis. Primary antibodies for immunoblot detection were purchased from Cell Signaling Technology: NOTCH1 XP (#3608S) and cleaved NOTCH1 (#4147S). Loading controls were performed with antibodies specific for β-Actin (#3700S and #4970S).

IRDye 680LT Goat anti-Mouse IgG (#925-68020, LI-COR Biosciences, Lincoln, NE, USA) and IRDye 800CW Goat anti-Rabbit IgG (#925-32211, LI-COR Biosciences) were used as secondary species-specific antibodies. Membranes were detected using the LI-COR Odyssey imaging system (LI-COR Biotechnology) and the Chemidoc MP Imaging System (Bio-Rad Laboratories).

### 3.6 Cell Treatment and Viability Assays

CAD204520 was obtained as a kind gift from WDB R&D Consulting (Denmark). Venetoclax (S8048) and ibrutinib (S2680) were purchased from Selleck Chemicals (SelleckChem, Houston, TX, USA) and dissolved in DMSO, according to manufacturer's instructions. A total of 40.000 cells were arrayed in 96-well plates (Greiner Bio-One, Belgium) in a volume of 100 µL per well using the MultiDrop Combi Reagent Dispenser (Thermo Scientific). Cells treatment was performed with Tecan D300e (Tecan Group, Zurich, Switzerland). ATP-based cell viability was determined using the CellTiter-Glo viability assay (#G7573, Promega Corporation) after 72 hours treatment. Luminescence was measured using a Victor X4 (Perkin Elmer). Values for IC50 and the area under the curve (AUC) were calculated using GraphPad Prism 9 software (La Jolla, CA, USA). Death rate of cells after CAD204520 treatment was assessed using a flow cytometric assay, using the Attune NxT flow cytometer (Thermo Fisher Scientific). Cells were stained with Propidium iodide (PI) (#40017, Biotium, Inc. Landing Parkway Fremont, CA, USA) and human CD5 (#345781, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The percentage of death cells was quantified acquiring a minimum of 10.000 events. Data were processed with FlowJo V10 (Tree Star, LLC, Ashland, OR, USA) analytical software.

### 3.7 Drug Combination Treatment and Synergy Assessment

Cell solution (50 µL/well of 0.02 x $10^6$/ml) was dispensed in 384-well plates (#3570, Corning Life Sciences Plastic, Bedford, MA, USA) using Multidrop™ Combi (#5840300, Thermo Fisher Scientific). Venetoclax, ibrutinib, CAD204520 were dissolved in DMSO and added with a nanometric Tecan D300e dispenser. We tested ibrutinib and venetoclax both individually and in combinations for a total of twenty-five combinatorial points in three CLL primary samples. Each drug was tested in 5 concentrations, with or without 2 µM of CAD204520. Cell viability was assessed after 72 hours of drug treatment using a CellTiter-Glo ATP assay. Analysis was performed with Combenefit MATLAB R201[92], using the HSA synergy analysis. A color scale bar represents the level of drugs antagonism or synergism. For the 3-drugs combination, we assessed the synergy, using three primary CLL samples. Each drug was tested at five different concentrations. Cell viability was assessed after 72 hours of drug treatment using a CellTiter-Glo ATP assay. Subsequently, data were imported into R (version 4.3.1). Before calculating synergy scores, we addressed situations where some viability values exceeded 1 (or 100%), as such instances can pose issues when computing synergy scores.

Therefore, viability data was re-scaled to set the maximum value to 1 without altering the minimum value in each sample using a generalized scaling formula. Moreover, this adjusted viability was converted into inhibition which was then used to compute three different harmonized synergy scores: HSA, Bliss and ZIP implemented in the SynergyFinder Plus R package[93, 94]. Each harmonized score is centered on zero with positive values pointing towards synergistic effect and negative values pointing to antagonistic effect, therefore all scores can be compared to each other. Then, we computed a linear fold-change between combinations of 3 drugs (venetoclax-ibrutinib-CAD204520) and 2

drugs (venetoclax-ibrutinib) in order to get a quantitative measure of the gain/loss induced by the addition of varying doses of CAD204520 to the venetoclax-ibrutinib combinations. A positive fold-change value represents combinations in which the addition of CAD204520 caused an inhibition improvement while negative fold-change value represents combinations in which the addition of CAD204520 resulted in inhibition dampening. All plots were made using R and the ggplot2 package[95].

### 3.8 Cell Competition Assay

REC-1 were transduced with a green fluorescent protein (GFP) lentiviral expressing vector and co-cultured with JEKO-1 cells in a 1:1 ratio in RPMI 1640, 10% FBS, 1% P/S, and 1% MEM Non-Essential Amino Acids. Three million cells per condition were treated with vehicle or CAD204520 at concentration of 2 and 4 μM. Cells were incubated at 37°C for 72 hours, washed in PBS and then stained with PI and human CD5 for 15 min. Fluorescent signal was detected by flow cytometry and a minimum 10.000 events were collected for each biological condition. Data were processed by FlowJo V10 analytical software.

### 3.9 *In vivo* Study

Ten non-irradiated 6 to 7 weeks old non-obese diabetic (NOD)-scid IL2rγ(null) (NSG) mice (Charles River Laboratories, Wilmington, MA, USA) were used for the *in vivo* study. Ten million of REC-1 cells and JEKO-1 cells, dissolved in 250 μL saline solution, were subcutaneously injected in the left and right flank of the same mouse, respectively. Once the tumor was established and palpable on both sides, mice were divided into vehicle and CAD204520 treatment groups, respectively. NSG mice received 45 mg/kg CAD204520

(Tween80 0.5% w/v; hydroxypropyl methylcellulose (HPMC) 1% w/v) or vehicle by oral gavage from day 1 to day 5 and from day 8 to day 12. Weight was monitored every 2 days. The anti-tumor activity of CAD204520 was assessed by measuring the REC-1 and JEKO-1 tumor volume by caliper measurement at days 0, 3, 6, 8, and 10, and by quantification of NOTCH1 (#PA5-99448; CleavedVal1744; Invitrogen) and KI-67 (#R626; Agilent, Santa Clara) positive cells in formalin-fixed, paraffin-embedded tumor sections. Images were acquired at different magnifications using a Leica DM750 microscope (Leica Microsystems, Wetzlar, Germany). The studies were carried out under an approved protocol n°682/2019-PR at the University of Parma.

# 4. RESULTS

## 4.1 Patients cohort characterization

Isolated PEST mutations most frequently occur in CLL and MCL, as such they represent an ideal model to investigate the development of Notch1 targeted therapies. From October 2021 to August 2022, we collected 37 leukemic cells samples from patients affected by CLL. The cohort included 21 males and 16 females (male to female ratio: 1.31) with a median age of 73 years (range: 46-94). All cases were classified according to the Rai-Binet staging classification, the CLL-international prognostic index (CLL-IPI) and characterized by genetic and molecular genomics approaches including cytogenetics, fluorescence in situ hybridization (FISH), immunoglobulin heavy chain variable (*IGHV*) gene mutation status, and mutational fingerprint by next-generation sequencing (NGS) (Figure 2A). The median lymphocyte count was 54,460/μL (range: 10,070-286,000/μL). Twenty-nine patients out of thirty-seven (78.3%) were treatment-free, seven (18.9%) received one line of therapy and three were undergoing ibrutinib treatment at the time of sample collection. We sequenced 54 genes involved in lymphomagenesis and, this analysis revealed a frequency of *NOTCH1* mutations in 16% of cases, 6 out 37, in line with previous findings[96]. We observed 5 different *NOTCH1* mutations in the exon 34, encoding for the PEST domain (Figure 2B). Two patients carried a p.(Pro2514Argfs*4) mutation, one exhibited a p.(Val2473), one a p.(Gln2440del), and one a mutation occurring in the ankyrin repeats motif p.(Gln2123*). These mutations are common in this disease [50]. We also recorded a rarer three nucleotide deletion, p.(Met2363del), occurring in the transactivation domain (TAD) and causing a frameshift deletion. A CLL patient showed a *FBXW7* mutation p.(Met404Ilefs*3) expected to increase N1-ICD half-life[97]. The most recurrent mutations were observed in the *CHD2*, *ARID1A*,

*KMT2A,* and *KMT2D* genes with frequencies of 56.7%, 43.2%, 35.1%, and 32.4%, respectively. Additionally, we identified the presence of other unfavorable mutations in the *BIRC3* and *SF3B1* genes, both at a frequency of 13.5%. Cytogenetic testing was examined on 7 patients (18.9%), three of whom exhibited a complex karyotype. According to the FISH analysis, deletions of 13q, 17p, 11q, and trisomy of chromosome 12 were found in 64.8%, 18.9%, 13.5%, and 13.5% of cases, respectively (Figure 2C). *TP53* mutations affected two out of thirty-seven patients (5.4%) in our series, consistent with previous report[91, 98, 99]. Additionally, two other patients exhibited a minor *TP53* mutated subclone with variant allele fraction (VAF) of 4.5 and 7.7%, respectively. Furthermore, in one of these cases, there was a concurrent presence of del(17p).

Previous studies have suggested that the *NOTCH1* activation, monitored by a monoclonal antibody that targets N1-ICD may occurs also in the absence of a gain-of-function mutation[100]. In our cohort, this appears to be true in 67% of the cases, confirming the presence of samples with no detectable mutation by NGS but with N1-ICD expression and suggesting differences in the turnover of the intracellular domain (Figure 2D).

### 4.2 CAD204520 suppresses leukemia growth and Notch1 signaling in PEST mutated cells

To test the antiproliferative effect of CAD204520 in PEST mutated lymphoproliferative disorders we used the following cell line models: T-ALL (ALL-SIL, CTV-1, SKW-3/KE-37), MCL (REC-1, JEKO-1, Granta-519) and CLL (MEC-1). Cell lines carrying PEST domain mutations, such as CTV-1, SKW-3/KE-37 and REC-1, showed N1-ICD expression compared to the WT ones (Figure 3A).

SERCA inhibitors block Notch trafficking and impede Notch proteins delocalization on the cellular surface[81, 82]. This effect can be monitored by several approaches including immunofluorescence analysis and western blotting[81]. SERCA inhibitors are expected to decrease the level of the NOTCH1 transmembrane subunit (N1-TM) (~110 kDa) while accumulating the unprocessed NOTCH1 (N1-FL) (~270 kDa) polypeptides in the endoplasmic reticulum. Consistently, PEST mutated tumors confirmed this hypothesis, as showed in Figure 3B. If Notch signaling sustains the growth of leukemia cells, its inhibition ultimately leads to a decrease in cellular proliferation and cell death. We compared PEST mutated tumor models (CTV-1, SKW-3/KE-37, and REC-1) to WT ones (JEKO-1, Granta-519, MEC-1) and showed that cell lines carrying the PEST mutation were more sensitive to CAD204520 inhibition (Figure 3C,D) with increasing apoptosis after treatment (Figure 3E-G).

We next extended these observations to clinical samples carrying *NOTCH1* mutations. In these samples, we confirmed an increased level of sensitivity in cells with mutations compared to WT (Figure 3H). N1-FL accumulates, N1-TM and N1-ICD decreased upon CAD204520 treatment in mutated cells according to the mechanism described above (Figure 3I). We also observed a difference in viability and anti-proliferative response to CAD204520 between WT and mutated samples through an ATP-based viability assay and flow cytometry analysis (Figure 3J).

Overall, our data suggest that CAD204520 inhibits *NOTCH1* PEST mutations in both cell lines and in primary CLL samples, retaining the advantageous anti-tumor effect on mutated over WT cells.

### 4.3 CAD204520 treatment exerts preferential anti-Notch1 efficacy in a xenograft lymphoma model

To further validate the activity of CAD204520 in *NOTCH1* PEST mutated tumors, we established a flow cytometry competition assay using two MCL cell lines (REC-1 and JEKO-1) characterized by opposite *NOTCH1* mutational status. The choice of this model is related to the absence of commercially available CLL cell lines carrying *NOTCH1* PEST mutation. We transduced the REC-1 cell line carrying *NOTCH1* PEST mutation with a green fluorescent protein (GFP) and we treated REC-1-GFP and JEKO-1, cultured in a 1:1 ratio, at different CAD204520 concentrations (Figure 4A). As shown in Figure 4B, mutated cells displayed a more pronounced effect of CAD204520 compared to WT cells, thereby replicating the findings described earlier in the clinical sample setting.

In the past, we demonstrated that short-term exposure to CAD204520 led to a reduction in leukemia burden in a preclinical T-ALL model in vivo[81]. Here, we aimed to expand upon this discovery by investigating its applicability in a B-cell lymphoma model. Additionally, we sought to confirm the safety profile of CAD204520 with prolonged administration. To achieve this, we have established a subcutaneous xenograft model for comparative analysis. JEKO-1 and REC-1 cells were injected in the left and right flank of the same mouse, respectively. A total of 5 mice per group received ten doses of vehicle or CAD204520 at 45 mg/kg by oral gavage. The administration was daily for 12 days including a two-day washout period after the initial 5 days of treatment (Figure 4C).

We observed a significant reduction in REC-1 tumor size and weight starting from six days after the start of treatment and at the end of the treatment, respectively, as illustrated in Figure 4D, E. Notably, the treatment exhibited excellent tolerability, with no major toxicities, including weight loss, as shown in Figure 4F. In PEST mutated tumors the immunohistochemical analysis of

tumor samples obtained from treated mice indicated a consistent reduction in N1-ICD expression and proliferation, as evidenced by KI-67 staining. In contrast, there was no significant change in NOTCH1 expression or proliferation rate in JEKO-1 tumors, as depicted in Figure 4G, H.

These promising preclinical in vivo model results, which align with our in vitro observations, strengthen our hypothesis that CAD204520 may be a potential candidate for improving the effectiveness of current drug treatments in patients with *NOTCH1* PEST mutations.

### 4.4 CAD204520 increases the effect of venetoclax-ibrutinib combination treatment in *NOTCH1* PEST mutated samples

Ibrutinib, an inhibitor of the Bruton tyrosine kinase (BTK), and venetoclax, an inhibitor of B-cell lymphoma-2 protein (BCL-2), were recently approved in combination by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for the treatment of adult patients with CLL, offering a potential standard treatment for CLL and hopefully for MCL patients in the future[101, 102]. However, it's important to note that CLL remains an incurable disease, and the presence of *NOTCH1* mutations is associated with an unfavorable outcome in both CLL and MCL patients.

For this reason, we have explored an approach involving the use of CAD204520 to enhance the response of the venetoclax-ibrutinib combination in patients carrying *NOTCH1* PEST mutations.

First, we treated both WT and *NOTCH1* mutated samples with increasing concentrations of ibrutinib and venetoclax. Subsequently, we added a constant concentration of CAD204520 (2 µM) to the previously treated cells. The combination with CAD204520 resulted in a synergistic effect, observed in the *NOTCH1* mutated samples, consistent with our prior findings (Figure 5A, B).

Next, we expanded the range of concentrations tested to assess the synergistic/antagonistic status of venetoclax-ibrutinib-CAD204520 combinations in a 3-drug synergistic assay. This produced more than 200 combinatorial points interpolated from 5 concentrations of each drug. To assess the contribution of CAD204520 to venetoclax-ibrutinib combinations, we computed three different harmonized synergy scores (highest single agent: HSA; Bliss; and zero interaction potency: ZIP) for three different primary samples (a WT and two *NOTCH1* mutated samples). *NOTCH1* PEST mutated samples showed a stronger synergistic signal when compared to WT in all synergy scores. Furthermore, non-mutated sample showed more combinations resulting in antagonistic effect pointing to a lower inhibitory strength of venetoclax-ibrutinib-CAD204520 (Figure 5C-E).

Although synergy scores can indicate whether a drug combination induces synergistic or antagonistic effect, they do not quantify the gain or loss of inhibition when comparing the 3-drugs combination (venetoclax-ibrutinib-CAD204520) to the 2-drugs combination (venetoclax-ibrutinib). Therefore, to further dissect the difference between *NOTCH1* PEST mutated and WT samples, we computed a linear fold-change between the inhibition percentage of venetoclax-ibrutinib-CAD204520 combinations and those of venetoclax-ibrutinib at the same concentrations. Both mutated samples showed a linear fold-change ≥ 1 (gain of inhibition) when venetoclax was ≤ 0.001 µM regardless of the ibrutinib dose (0.01 µM, 0.1 µM and 0.5 µM), especially when CAD204520 was in the 0.5-4 µM range. On the opposite side, the WT sample showed a fold-change close to zero, indicating the lack of difference in inhibition between combinations with and without CAD204520. In addition, the WT sample showed a negative fold-change (CAD204520 4 µM and venetoclax ≤ 0.001 µM) indicating a loss of inhibition consequently CAD204520 addition to venetoclax-ibrutinib combination (Figure 5F).

Our results support the idea that CAD204520 enhanced the effect of venetoclax-ibrutinib combinations synergy treatment in *NOTCH1* PEST mutates samples while in WT samples showed a very limited improvement.

# 5. DISCUSSION

Recent developments in cancer target therapy have shown promising results by targeting the genetic mutations or proteins responsible for tumor growth, leading to more effective and less toxic treatments. For example, patients with acute myeloid leukemia (AML) carrying the *FLT3* mutation may receive a targeted therapy like midostaurin or gilteritinib to inhibit the activity of the mutated FLT3 protein, potentially leading to improved outcomes and reduced side effects compared to traditional chemotherapy[103, 104]. This paradigm appears to hold promise for all the mutations occurring in enzyme regulation in hematopoietic differentiation[105, 106] and metabolism[107], but it is certainly less applicable to mutations involving transcription factors[108].

An example is the Notch signaling. *A priori*, NOTCH1 is not an ideal candidate for canonical drug targeting due to its involvement in various biological processes and cell types. This wide-ranging influence poses a potential limitation to develop effective anti-Notch1 therapies, as targeting of NOTCH1 in non-leukemic cells could lead to adverse and toxic effects[22]. However, this is not the case. *NOTCH1* has been established as an oncogenic driver in several tumor models such as T-ALL[43], CLL[109], MCL[51] and a wide range of solid tumors[22], where activation recurs in different phases of the disease, both at diagnosis and relapse[43, 110, 111]. Canonical Notch1 signaling requires, multiple proteolytic cleavages such as S1 by a furin-like convertase in the endoplasmic reticulum/*trans*-Golgi compartment[112, 113], a second cleavage (S2) within the NOTCH juxta-membrane extracellular domain mediated by zinc-dependent disintegrin and metalloprotease (ADAM10 or 17) at the membrane surface[114, 115] and finally a third cleavage (S3) mediated by the gamma-secretase complex[116]. These cleavages have the potential for

enzymatic inhibition. For example, well-known GSI compounds have been extensively investigated in clinical trials, yet with limited success thus far[88].

In addition to enzymatic targeting, three other approaches for Notch signaling inhibition are under exploration. The first involves blocking individual NOTCH receptors or ligands with targeted antibodies. For example, a humanized antibody targeting NOTCH1, OMP-52M51 (brontictuzumab), has demonstrated efficacy in the inhibition of DLL4-mediated cleaved-NOTCH1 overexpression in pre-clinical studies[117, 118] and has entered phase I trials for solid tumors and relapsed/refractory (R/R) lymphoid malignancies (NCT01778439, NCT01703572). The second approach relies on the binding inhibition of N1-ICD and its transcriptional complex[119]. The most advanced example in this case is CB-103, a small molecule investigated in Notch-driven cancers[120-122]. CB-103 showed a good efficacy in *NOTCH* mutated solid tumors and an acceptable safety profile in a phase I clinical trial[123]. The third approach depends on NOTCH1's requirement to undergo cellular trafficking before relocating to the nucleus and initiating a transcriptional signal. In this context, small molecules targeting SERCA[83] or other ion channels[124, 125] serve as prototypes for this therapeutic avenue. CAD204520, for example, showed an excellent safety profile, and a promising therapeutic index in preclinical models of T-ALL[81]. Previously, we collaboratively demonstrated that tumors with PEST mutations respond to ion channel modulators, such as the $Ca^{2+}/Na^{2+}$ pump inhibitor bepridil[125]. Thapsigargin, bepridil, ionomycin, salinomycin and others were all initially identified through a gene-expression-based screen[82, 126] designed to discover modulators of mutated Notch transcriptional programs in T-ALL. The repurposing effort of using these small molecules in tumors with *NOTCH1* mutations other than T-ALL, suggests that targeting Notch1 trafficking[86, 127] could be equally effective across various tumor types[85]. In this sense the activity of CAD204520 in CLL and MCL carrying PEST mutations does not surprise. However, what could not have

been predicted is the fact that these mutations enhance the sensitivity to SERCA inhibitors compared to WT cases both *in vitro* and *in vivo*.

Targeting PEST domain mutation in hematological malignancies has been investigated in a few studies. The GSI PF-03084014 induces apoptosis in leukemic CLL cells carrying *NOTCH1* mutations, an effect potentiated by fludarabine[128]. Similarly, CLL xenotransplant models treated with bepridil significantly reduced tumor infiltration with no remarkable toxicity nor activity on NOTCH2 WT protein[125]. In MCL, the only clinically tested compound is the monoclonal antibody brontictuzumab. However, preclinical activity of brontictuzumab in MCL cell lines was modest both *in vitro* and *in vivo* and similar to a minor clinical effect in MCL patient treated in the phase I study[129, 130]. No clinical trials evaluating GSI in MCL are currently ongoing.

Furthermore, none of the previously mentioned studies performed a direct comparison of the effects of Notch1 inhibitors in mutated and non-mutated models in a head-to-head study. Such a comparison is crucial, considering the involvement of WT Notch signaling in some tumor types[131]. Achieving WT inhibition will necessitate dose adjustments, different schedules, or combinatorial approaches to effectively target WT polypeptides.

Besides Notch1, several other signaling pathways and small molecules dominated the last ten years in research on cancer carrying PEST domain mutations. This is the case of B-cell receptor (BCR)-associated kinases, such as BTK, phosphoinositide 3-kinases (PI3K) and the anti-apoptotic protein BCL-2[132-135]. Randomized clinical trials demonstrated impressive activity of ibrutinib and novel BTK inhibitors for the treatment of R/R disease[136-138], del(17p) CLL patients[139] and *de novo* or R/R MCL patients[140, 141]. In parallel, venetoclax was the first BCL-2 inhibitor to enter routine clinical practice. In a phase I study, venetoclax induced durable responses in 79% of patients with R/R CLL, including complete remissions in 20% of patients[142]. Given their impressive effect, it is a reasonable strategy to combine the two molecules. In the setting

of combinatory therapy venetoclax plus ibrutinib, a phase II non-randomized trial (NCT02756897) in treatment-naive patients[101] has shown 3-years progression-free survival (PFS) of 93%, including durable activity in del(17p)/*TP53* mutated CLL. Interestingly, the combinatory treatment with ibrutinib plus venetoclax has shown encouraging clinical activity in early phase studies, reaching the phase III SYMPATICO trial with strong efficacy in patients with R/R MCL[102].

In the scenario where the majority of CLL patients respond to ibrutinib or venetoclax, *NOTCH1* mutated patients still represent an aggressive subgroup of the disease, as *NOTCH1* mutation showed to be an independent predictor of survival and Richter transformation[54, 143, 144]. Based on this assumption, one question arises: when and how to incorporate Notch inhibitors? One possible answer is to consider cases that have relapsed or are refractory to therapy, or cases that are progressing despite ongoing therapy[145]. Combination therapy appears to be a potential strategy, as there is evidence, for example, that GSI enhances the anti-leukemic activity of ibrutinib in CLL cells by down-regulating the Notch1 and c-Myc pathways[146]. In addition, ibrutinib treatment showed to downregulate NOTCH over time as part of downstream pathway of the BCR[147]. Finally, although the presence of a mutation does not appear to negatively impact the efficacy of ibrutinib in terms of disease progression outcomes[148], other findings correlate *NOTCH1* mutation with reduced redistribution of lymphocytosis and nodal shrinkage, responsible for partial responses and early relapses[149]. For MCL instead the answer is simpler given the urgent need of new approaches for R/R cases or cases not eligible for CAR-T therapy[150].

However, mimicking this complex setting requires building a feasible toolbox for the analysis of drug synergy with more of two compounds. This effort presents several challenges, for example the lack of tools capable of handling combinations involving N-drugs (N>2) which limits the information retrievable from these combination experiments. Another limiting factor involves the

strategy employed by current methods, where synergy scores for combinations of 3 drugs are computed by comparing the effect of the triplet with the effects of each single drug, without considering any comparison between the 2-drug combinations and the triplet[151, 152]. A method that would eliminate these issues involves computing synergy scores for pairwise combinations and using them to gain information about higher-order combinations. However, this type of approach has been reported to rarely show synergy, whereas antagonism is more common[153-156]. This arises from the inability of pairwise comparisons to predict higher-order interactions[152].

To overcome existing analytical limitations, we decided to use established, but methodologically limited, synergy scores (HSA, Bliss, and ZIP). Additionally, to obtain a direct measure of gain or loss of inhibition we integrated the results with the implementation of a simple quantitative method based on the linear fold-change between venetoclax-ibrutinib-CAD204520 and venetoclax-ibrutinib combinations. Collectively, our data suggests that low concentration of venetoclax may be sufficient to prime the cells to death when co-treated with the BTK inhibitor ibrutinib and the SERCA inhibitor CAD204520. This approach may reduce the requirement for higher concentrations of venetoclax, which can potentially give rise to BCL-2 resistant clones. The addition of CAD204520, in turn, can have a sustained positive impact on controlling the proliferation of leukemic cells.

# 6. CONCLUSION

In conclusion, despite remarkable improvement in the field of lymphoproliferative disorders, the treatment of diseases such as CLL and MCL is still challenging. The current strategy consists of a heterogeneous combination of approaches. From intense chemotherapy regimens to combinations with monoclonal antibodies, bispecific T-cell engagers (BiTE) and chimeric antigen receptor (CAR) T-cells, none of them seems to demonstrate a definitive solution, especially in patients with specific mutations.

In the era of target therapy and personalized medicine, our approach represents a new possible avenue for patients characterized by *NOTCH1* mutation.

We showed that targeting of Notch1 aberrant pathway through inhibition of SERCA is currently feasible. This finding has been observed previously in *NOTCH1*-mutated T-ALL and now confirmed in PEST-mutated lymphoproliferative diseases. We also confirmed a promising safety profile of CAD204520. In fact, our preclinical models showed a response in *NOTCH1*-mutant models respect to wild type, without relevant side effects.

Furthermore, our results on the synergistic effect of the use of CAD204520 with the first line CLL treatment venetoclax-ibrutinib could represent an important chance for patient characterized by *NOTCH1* mutation.

In summary, our work positions SERCA inhibitors as potential modulators of the Notch signaling characterized by PEST mutations such as CLL and MCL and supports the development of novel strategies with complex matrices of drug-drug combinations in preclinical cancer related studies.

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# 8. FIGURE LEGENDS

**Figure 1. NOTCH general protein structure and activation pathway**

A) The figure illustrates the general NOTCH structure.

B) Overview of the NOTCH signaling pathway.

**Figure 2. *NOTCH1* mutational status in chronic lymphocytic leukemia (CLL) primary samples and characteristics of the patient cohort**

A) The OncoPrint illustrates the distribution of gene mutations affecting individual samples. Single nucleotide polymorphisms (SNPs) are represented in red, and insertions/deletions (indels) are in blue. Each row in the OncoPrint displays the percentage distribution of relative gene mutations in the entire cohort (shown in the right histogram panel). Each column represents the total number of mutations for each patient, with a specific indication of the mutation type (upper histogram panel; red: SNP, blue: indel). The OncoPrint also provides relevant clinical, genetic, molecular, and prognostic characteristics of the patient samples collected for this study (lower panel).

B) The linear structure of the human NOTCH1 protein is depicted, with each colored block representing an exon. The PEST domain illustrates the distribution of PEST mutations found in CLL patient samples.

C) Patient characteristics for the collected CLL primary samples.

D) Western immunoblotting results display the expression of unprocessed full-length NOTCH1 precursor (FL), furin-processed NOTCH1 transmembrane

subunit (TM), and cleaved intracellular domain (ICD) in CLL primary samples. β-Actin serves as the loading control. NOTCH1 mutated patient samples are indicated in light red.

**Figure 3. CAD204520 inhibits Notch1 signaling and impairs cell growth in PEST mutated lymphoproliferative malignancies**

A) Protein expression of NOTCH1 processed isoforms in a panel of T-ALL, MCL, and CLL cell lines. β-Actin was used as a loading control. (FL: full-length unprocessed precursor; TM: transmembrane; ICD: intracellular domain). The table shows *NOTCH1* mutational status in the cell lines.

B) Effect of CAD204520 treatment for 24 hours on Notch1 trafficking and activation in cell lines (CTV-1, SKW-3/KE-37, REC-1) with PEST domain mutations. β-Actin was used as a loading control.

C) Effect of CAD204520 treatment on cell viability after 72 hours in *NOTCH1* PEST mutated (REC-1, SKW-3/KE-37, CTV-1) and *NOTCH1* WT (JEKO-1, Granta-519, MEC-1) cell lines. Error bars denote ± SD of a minimum of two replicates.

D) Comparison of the area under the curve (AUC) values after CAD204520 treatment of *NOTCH1* mutated and WT cell lines. Statistical significance was determined by a non-parametric t-test (* $p < 0.05$).

E) Effect of CAD204520 treatment on the induction of apoptosis. Annexin V/propidium iodide staining of MCL cells after 48 hours of treatment with the indicated concentrations of CAD204520. A minimum of 20,000 events was collected for each condition.

F) Western immunoblot showing the expression of cleaved PARP in *NOTCH1* WT (JEKO-1) and mutated (SKW-3/KE-37 and REC-1) cell lines treated at the indicated concentrations of CAD204520 for 24 hours. β-Actin was used as a loading control.

G) Densitometric quantification of indicated proteins in JEKO-1, SKW-3/KE-37, and REC-1 cells treated with indicated doses of CAD204520, as in Figure 3F.

H) Combined scatter and bar plot representing the AUC values of CAD204520 treatment in CLL primary samples with or without Notch1 activating pathway mutations. Statistical significance was determined using a non-parametric t-test (** $p < 0.01$).

I) Effect of CAD204520 treatment after 24 hours on Notch1 trafficking in CLL primary samples. β-Actin was used as a loading control.

J) Histogram plots showing the percentage of live and dead cells in 6 different samples (top: 3 *NOTCH1* WT samples; bottom: 3 *NOTCH1* mutated samples) after 72 hours of treatment with CAD204520 at indicated concentrations. Results were obtained with a luminescence-based and a flow cytometric assay, respectively. Error bars denote the SD of a minimum of two replicates. Statistical significance among groups was determined by a one-way ANOVA using Dunnett's correction for multiple comparison testing.

**Figure 4. CAD204520 preferentially inhibits cells with *NOTCH1* PEST mutations**

A) Outline of the cell-based competition assay: REC-1 cells were transduced with green fluorescent protein (GFP). REC-1-GFP+ cells were sorted and co-

cultured in a 1:1 ratio with JEKO-1 cells, then treated with CAD204520 at various concentrations for 72 hours.

B) Normalized effects of CAD204520 on cell viability in co-cultured REC-1-GFP+ and JEKO-1 cells treated for 72 hours. Statistical significance was determined by a two-way ANOVA. Error bars represent ± SD of a minimum of three replicates.

C) Design of the *in vivo* CAD204520 study: ten NSG mice were subcutaneously injected with REC-1 cells in the left flank and JEKO-1 cells in the right flank. On day 0, mice were randomized into two groups, with the former receiving CAD204520 at 45 mg/kg (day 1-5 "on"; day 6-7 "off"; day 8-12 "on") via oral gavage, and the latter receiving the vehicle.

D) Effect of CAD204520 administration on JEKO-1 and REC-1 tumor size fold change at different time points (mean ± SD of the five different mice treated with the vehicle or CAD204520). Statistical significance was determined using a non-parametric t-test (*$p < 0.05$; ** $p < 0.01$).

E) Effect of CAD204520 administration on JEKO-1 and REC-1 tumor weight at sacrifice (mean ± SD of the five different mice treated with the vehicle or CAD204520). Statistical significance was determined using a non-parametric t-test (*$p < 0.05$).

F) Effect of daily administration of 45 mg/kg of CAD204520 or the vehicle on body weight.

G) Immunohistochemical analysis of REC-1 and JEKO-1 tumor masses in the murine model treated with the vehicle or CAD204520 at 45 mg/kg for 10 administrations. The tumor masses from all mice were examined. Formalin-fixed, paraffin-embedded tissue sections were stained with NOTCH1 and KI-

67 antibodies. Scale bars: 50 μm. Representative results for one control animal and one CAD204520-treated animal are shown.

H) Quantification of NOTCH1 and KI-67 protein expression of immunohistochemical analysis showed in Figure 4G.

**Figure 5. CAD204520 increases the effect of venetoclax-ibrutinib treatment in *NOTCH1* PEST mutated samples**

A) Volcano surface plots of primary CLL samples with *NOTCH1* WT (CLL #2) and *NOTCH1* PEST mutation (CLL #20 and CLL #25) treated with venetoclax and ibrutinib. Each point represents an independent measurement. The plots illustrate the HSA analysis generated using the Combenefit script in MATLAB R201. The colorimetric scale represents the level of drug antagonism or synergism.

B) Volcano surface plots of primary CLL samples with *NOTCH1* WT (CLL #2) *NOTCH1* PEST mutation (CLL #20 and CLL #25) treated with venetoclax and ibrutinib plus 2 μM of CAD204520. Each point represents an independent measurement. The plots illustrate the HSA analysis generated using the Combenefit script in MATLAB R201. The colorimetric scale represents the level of drug antagonism or synergism.

C) Circular plots of 3-drug combinations in a *NOTCH1* WT primary sample (CLL#2) and two *NOTCH1* mutated primary samples (CLL#20 and CLL#25). The innermost rings represent the 5 drug concentrations in 3 color gradients (CAD204520 = yellow, venetoclax = cyan, ibrutinib = pink). The fourth ring represents the effect in terms of inhibition percentage for any given combination. The outermost ring represents a harmonized synergy score performed with the Bliss model. Red indicates a positive synergistic score

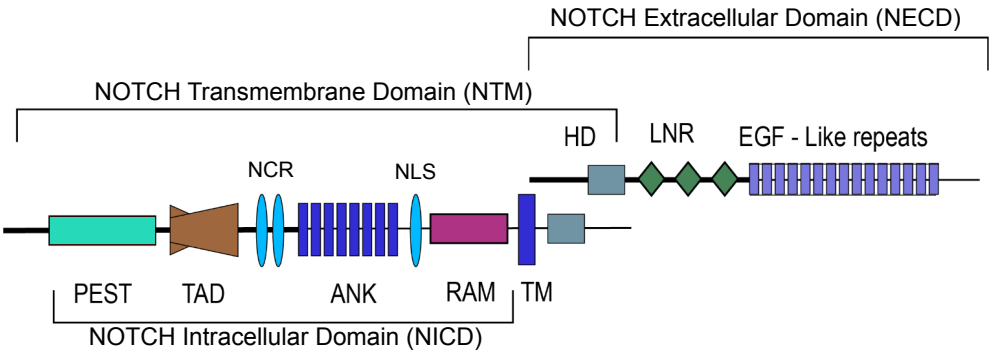
(pointing towards synergy), while blue represents a negative score (pointing towards antagonism). Zero indicates the absence of interaction.

D) Circular plots of 3-drug combinations following the same format as described in (C). The outermost ring represents a harmonized synergy score performed with the HSA model.

E) Circular plots of 3-drug combinations following the same format as described in (C). The outermost ring represents a harmonized synergy score performed with the ZIP model.

F) Heatmaps of samples CLL #2, CLL #20, and CLL #25 (from left to right) displaying the linear fold-change between combinations of venetoclax-ibrutinib-CAD204520 (columns) compared to venetoclax-ibrutinib combinations (rows). Each cell contains the fold-change between the 3-drug combination effect and the 2-drug combination effect at the same doses of the first 2 drugs. A positive fold-change indicates a gain in inhibition of the 3-drug combination compared to the 2-drug combination, while a negative fold-change indicates a loss of inhibition of the 3 drugs compared to the combination without CAD20452

A


NOTCH Extracellular Domain (NECD)
NOTCH Transmembrane Domain (NTM)
HD
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NOTCH Intracellular Domain (NICD)

B

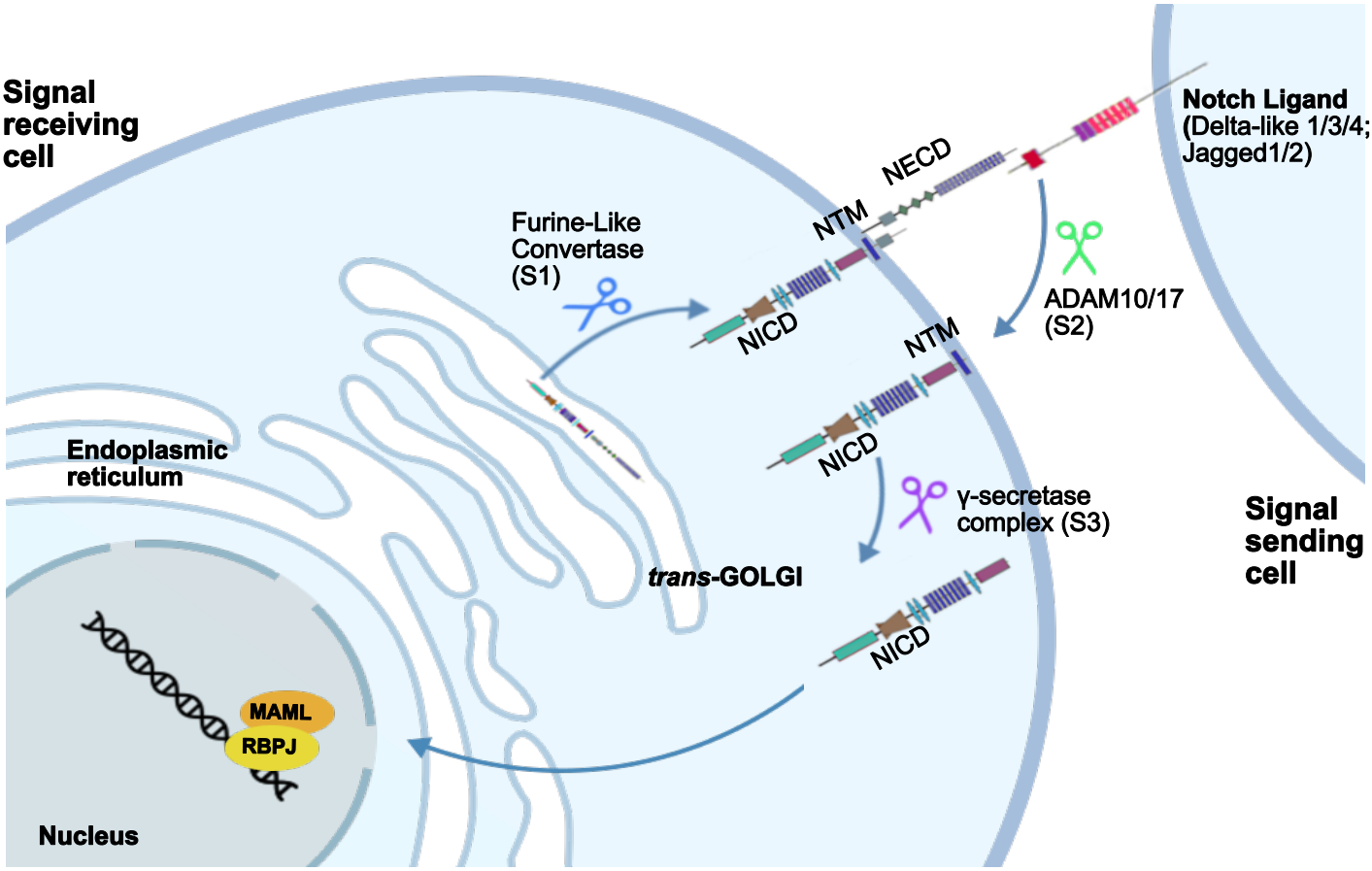
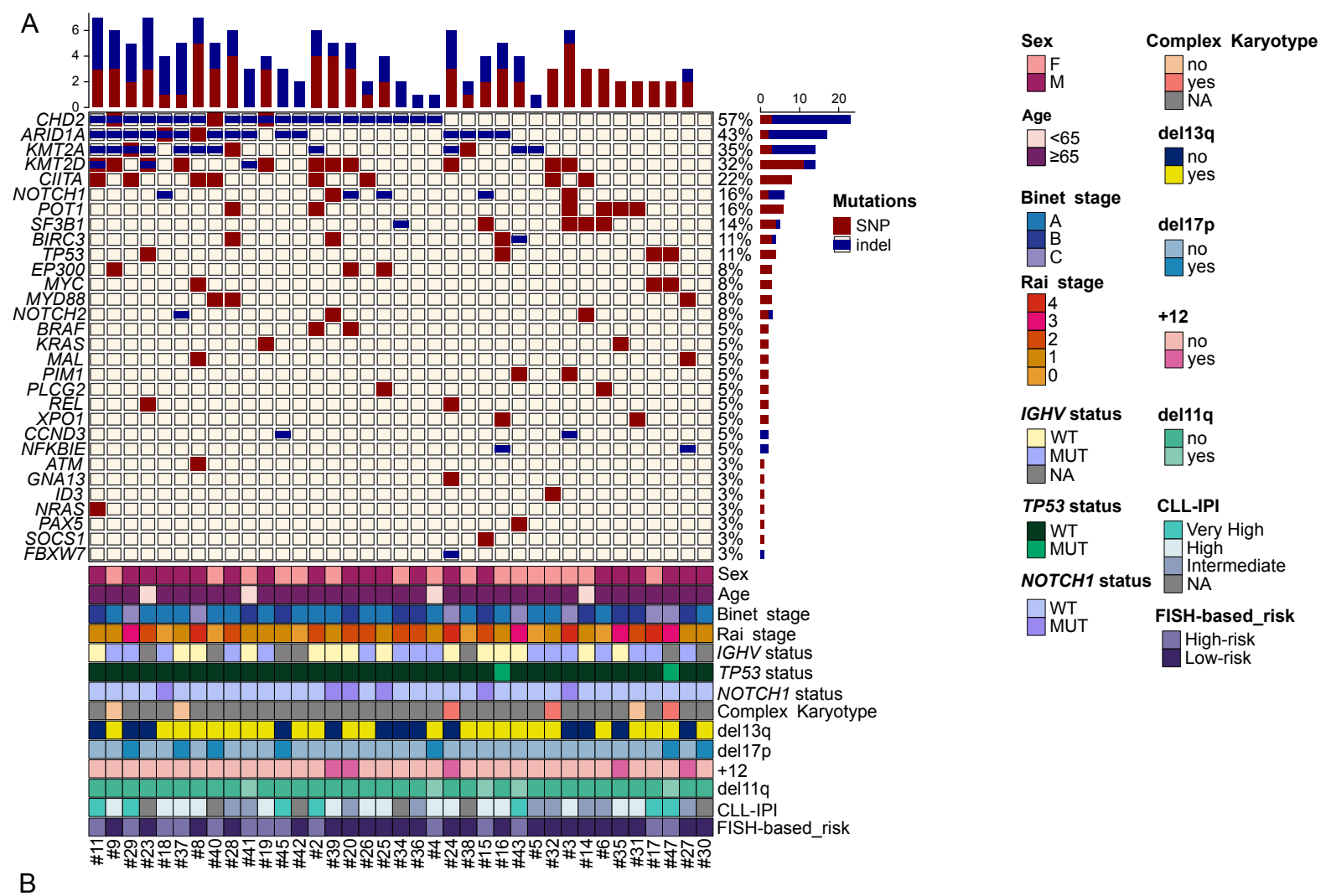
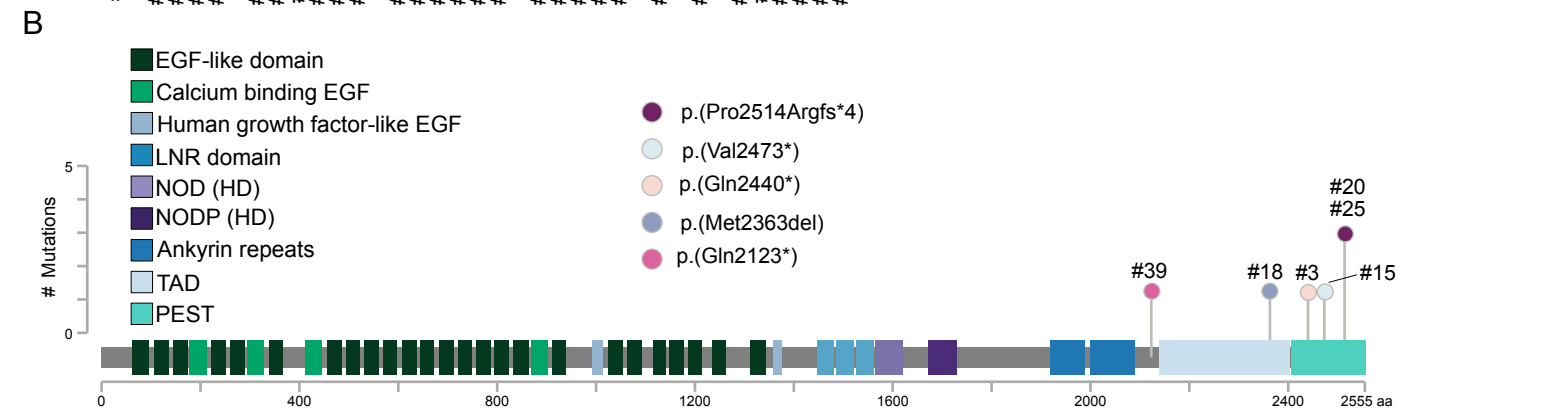

Signal receiving cell
Notch Ligand (Delta-like 1/3/4; Jagged1/2)
NECD
NTM
NICD
Furine-Like Convertase (S1)
ADAM10/17 (S2)
NTM
NICD
Endoplasmic reticulum
γ-secretase complex (S3)
Signal sending cell
trans-GOLGI
NICD
MAML
RBPJ
Nucleus

Figure 1




C

| Patient cohort (n = 37) | |
|---|---|
| **Age** | |
| Median | 73 |
| Range | 46-94 |
| **Sex (%)** | |
| Male | 21 (56.7) |
| Female | 16 (43.2) |
| M:F ratio | 1.31 |
| **Diagnosis (%)** | |
| CLL | 36 (97.2) |
| SLL | 1 (2.7) |
| **Stage Rai (%)** | |
| 0 | 6 (16.2) |
| 1 | 14 (37.8) |
| 2 | 9 (24.3) |
| 3 | 4 (10.8) |
| 4 | 4 (10.8) |
| **Stage Binet (%)** | |
| A | 19 (51.3) |
| B | 11 (29.7) |
| C | 7 (18.9) |
| **CLL-IPI (%)** | |
| Intermediate | 9 (24.3) |
| High | 15 (40.5) |
| Very High | 7 (18.9) |
| N/A | 6 (16.2) |

| | |
|---|---|
| ***IGHV* status (%)** | |
| Mutated | 17 (45.9) |
| Unmutated | 13 (35.1) |
| N/A | 7 (18.9) |
| **Cytogenetic/FISH abnormalities (%)** | |
| 13q deletion | 24 (64.8) |
| 17p deletion | 7 (18.9) |
| 11q deletion | 5 (13.5) |
| Trisomy 12 | 5 (13.5) |
| Karyotype available | 7 (18.9) |
| Complex Karyotype (≥3 abn.) | 2 (5.4) |
| Complex Karyotype (≥5 abn.) | 1 (2.7) |
| ***TP53* gene status (%)** | |
| Wild Type | 35 (94.6) |
| Mutated | 2 (5.4) |
| ***NOTCH1* gene status (%)** | |
| Wild Type | 31 (83.7) |
| Mutated | 6 (16.2) |
| ***FBXW7* gene status (%)** | |
| Wild Type | 36 (97.2) |
| Mutated | 1 (2.7) |
| ***BIRC3* gene status (%)** | |
| Wild Type | 32 (86.4) |
| Mutated | 5 (13.5) |
| ***SF3B1* gene status (%)** | |
| Wild Type | 32 (86.4) |
| Mutated | 5 (13.5) |

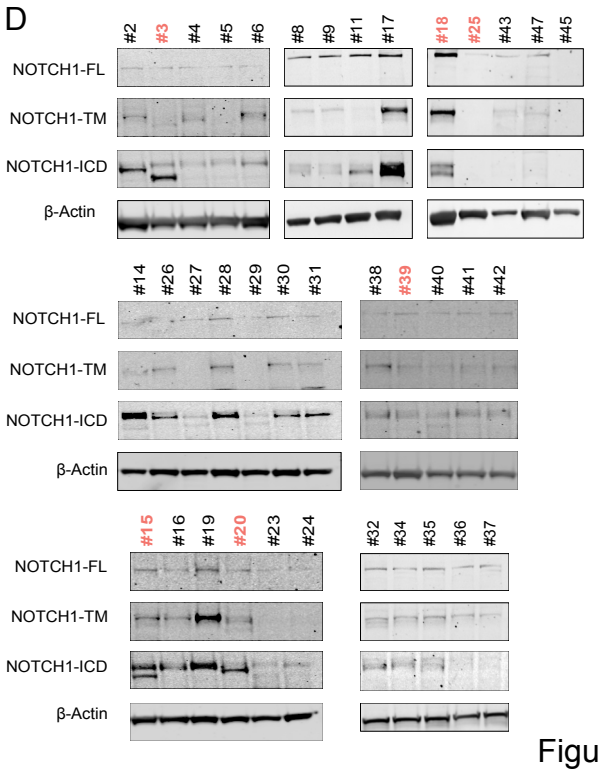



Figure 2

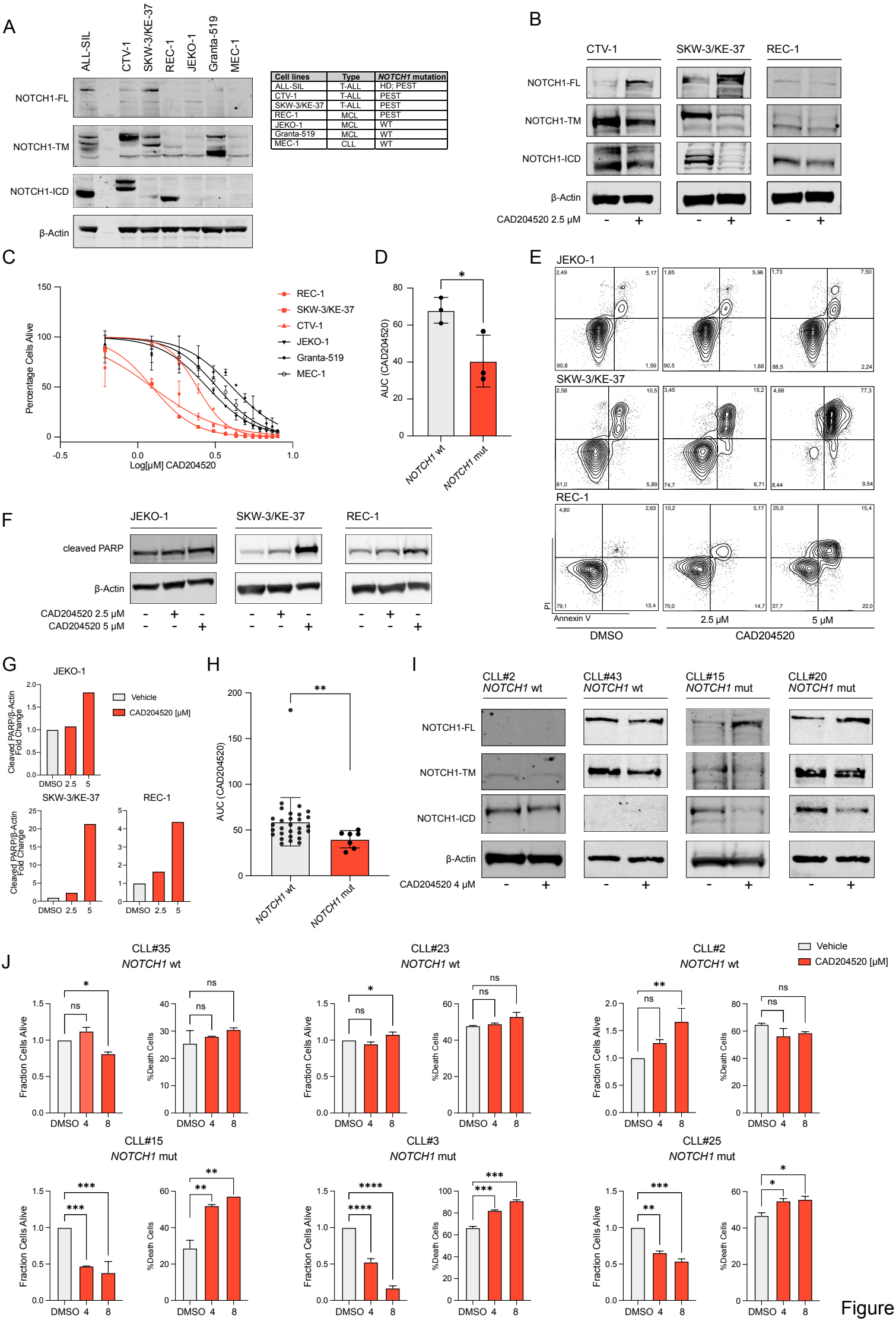

A
ALL-SIL
CTV-1
SKW-3/KE-37
REC-1
JEKO-1
Granta-519
MEC-1
NOTCH1-FL
NOTCH1-TM
NOTCH1-ICD
β-Actin
Cell lines | Type | NOTCH1 mutation
ALL-SIL | T-ALL | HD; PEST
CTV-1 | T-ALL | PEST
SKW-3/KE-37 | T-ALL | PEST
REC-1 | MCL | PEST
JEKO-1 | MCL | WT
Granta-519 | MCL | WT
MEC-1 | CLL | WT
B
CTV-1
SKW-3/KE-37
REC-1
NOTCH1-FL
NOTCH1-TM
NOTCH1-ICD
β-Actin
CAD204520 2.5 μM
C
Percentage Cells Alive
Log[μM] CAD204520
REC-1
SKW-3/KE-37
CTV-1
JEKO-1
Granta-519
MEC-1
D
AUC (CAD204520)
NOTCH1 wt
NOTCH1 mut
E
JEKO-1
SKW-3/KE-37
REC-1
PI
Annexin V
DMSO
2.5 μM
5 μM
CAD204520
F
JEKO-1
SKW-3/KE-37
REC-1
cleaved PARP
β-Actin
CAD204520 2.5 μM
CAD204520 5 μM
G
JEKO-1
Vehicle
CAD204520 [μM]
Cleaved PARP/β-Actin Fold Change
SKW-3/KE-37
REC-1
DMSO 2.5 5
H
AUC (CAD204520)
NOTCH1 wt
NOTCH1 mut
I
CLL#2 NOTCH1 wt
CLL#43 NOTCH1 wt
CLL#15 NOTCH1 mut
CLL#20 NOTCH1 mut
NOTCH1-FL
NOTCH1-TM
NOTCH1-ICD
β-Actin
CAD204520 4 μM
J
CLL#35 NOTCH1 wt
CLL#23 NOTCH1 wt
CLL#2 NOTCH1 wt
Vehicle
CAD204520 [μM]
CLL#15 NOTCH1 mut
CLL#3 NOTCH1 mut
CLL#25 NOTCH1 mut
Fraction Cells Alive
%Death Cells
DMSO 4 8

Figure 3

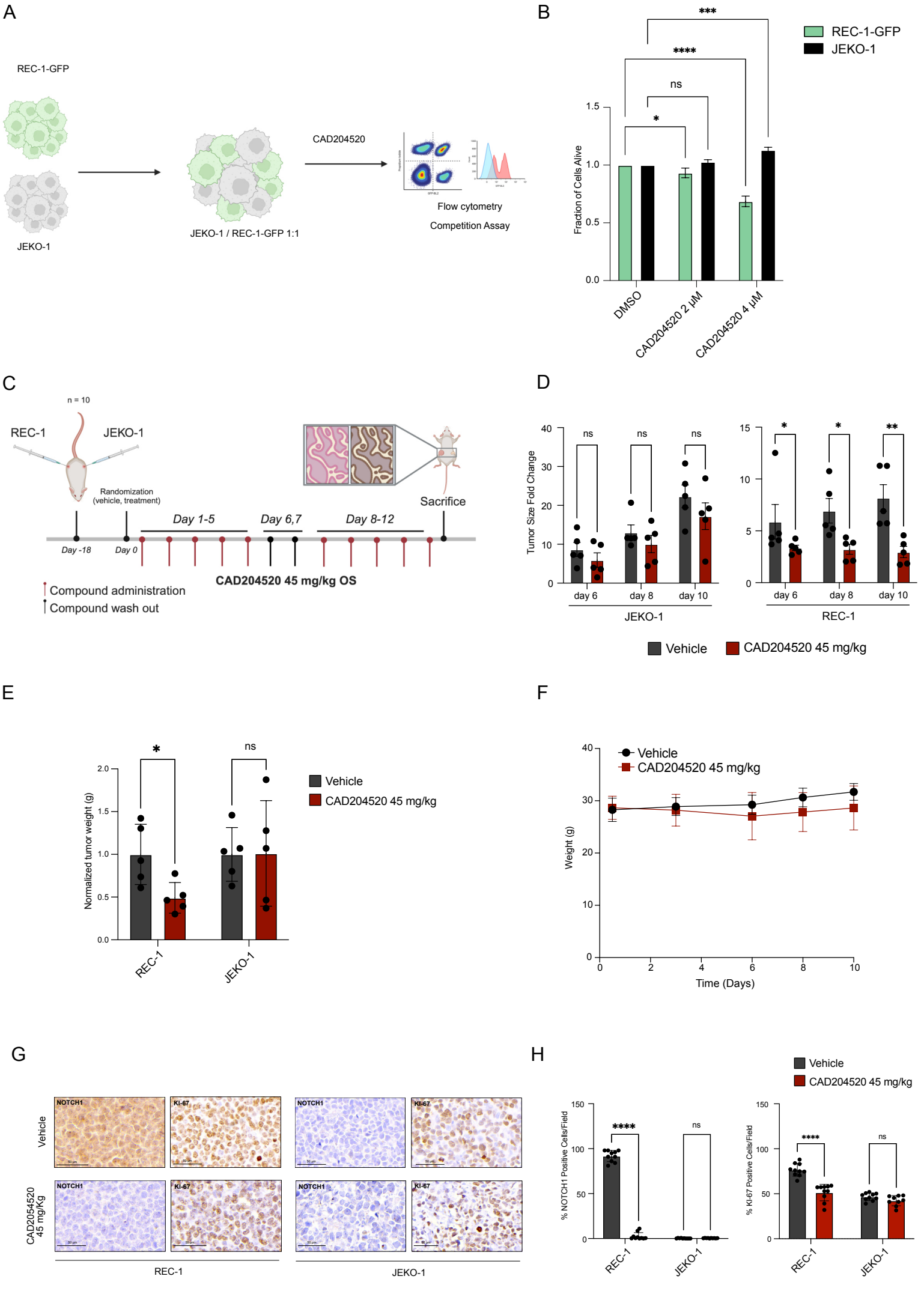

A
REC-1-GFP
JEKO-1
JEKO-1 / REC-1-GFP 1:1
CAD204520
Flow cytometry
Competition Assay
B
REC-1-GFP
JEKO-1
***
****
ns
*
Fraction of Cells Alive
1.5
1.0
0.5
0.0
DMSO
CAD204520 2 µM
CAD204520 4 µM
C
n = 10
REC-1
JEKO-1
Randomization
(vehicle, treatment)
Sacrifice
Day 1-5
Day 6,7
Day 8-12
Day -18
Day 0
CAD204520 45 mg/kg OS
Compound administration
Compound wash out
D
Tumor Size Fold Change
ns
ns
ns
*
*
**
day 6
day 8
day 10
JEKO-1
REC-1
Vehicle
CAD204520 45 mg/kg
E
*
ns
Normalized tumor weight (g)
2.0
1.5
1.0
0.5
0.0
REC-1
JEKO-1
Vehicle
CAD204520 45 mg/kg
F
Vehicle
CAD204520 45 mg/kg
Weight (g)
Time (Days)
G
Vehicle
CAD2054520
45 mg/Kg
NOTCH1
KI-67
REC-1
JEKO-1
H
Vehicle
CAD204520 45 mg/kg
% NOTCH1 Positive Cells/Field
% KI-67 Positive Cells/Field
****
ns
REC-1
JEKO-1

Figure 4

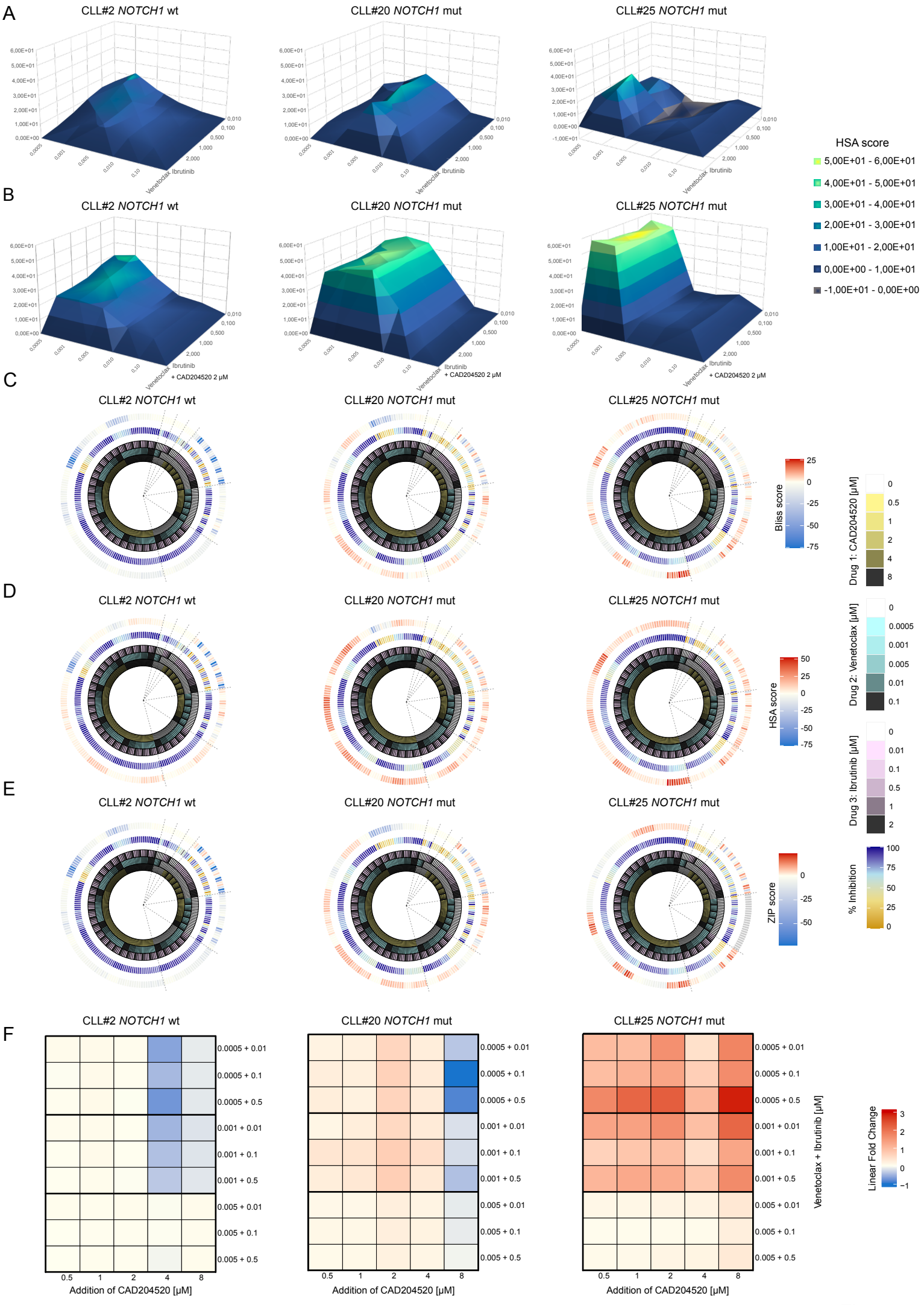

A
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
Venetoclax
Ibrutinib
HSA score
5,00E+01 - 6,00E+01
4,00E+01 - 5,00E+01
3,00E+01 - 4,00E+01
2,00E+01 - 3,00E+01
1,00E+01 - 2,00E+01
0,00E+00 - 1,00E+01
-1,00E+01 - 0,00E+00
B
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
Ibrutinib
+ CAD204520 2 µM
C
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
Bliss score
Drug 1: CAD204520 [µM]
D
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
HSA score
Drug 2: Venetoclax [µM]
Drug 3: Ibrutinib [µM]
E
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
ZIP score
% Inhibition
F
CLL#2 NOTCH1 wt
CLL#20 NOTCH1 mut
CLL#25 NOTCH1 mut
0.0005 + 0.01
0.0005 + 0.1
0.0005 + 0.5
0.001 + 0.01
0.001 + 0.1
0.001 + 0.5
0.005 + 0.01
0.005 + 0.1
0.005 + 0.5
Addition of CAD204520 [µM]
Venetoclax + Ibrutinib [µM]
Linear Fold Change

Figure 5