Pharmacological disruption of the Notch transcription factor complex

Rajwinder Lehali,1b, Jenela Zaric,1c, Michele Vigoloab,1, Charlotte Urechb,1, Viktoras Frismantasc,d, Nadine Zanggera,1, Linlin Caoab, Adeline Berger,1, Irene Chicote,9, Sylvain Loubéry,1, Sung Hee Choi,1, Ute Kocha, Stephen C. Blacklowl, Hector G. Palmere,1, Beat Bornhäuserc,d, Marcos González-Gaitánh, Yvan Arsenijevic,1, Vincent Zoetee,1, Jon C. Aste,1, Jean-Pierre Bourquinodd, and Freddy Radtkeb,1

1Swiss Institute for Experimental Cancer Research, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland; 2Research & Development, Cellestia Biotech SA, 4057 Basel, Switzerland; 3Department of Oncology, University Children’s Hospital Zürich, 8032 Zürich, Switzerland; 4Children’s Research Center, University Children’s Hospital Zürich, 8032 Zürich, Switzerland; 5Bioinformatics Core Facility, Swiss Institute of Bioinformatics, 1015 Lausanne, Switzerland; 6Unit of Gene Therapy and Stem Cell Biology, Department of Ophthalmology, Jules-Gonin Eye Hospital, University of Lausanne, 1004 Lausanne, Switzerland; 7Stem Cells and Cancer Group, Vall d’Hebron Institute of Oncology, 08035 Barcelona, Spain; 8Department of Biochemistry, Faculty of Sciences, University of Geneva, 1211 Geneva, Switzerland; 9Department of Biological Chemistry and Molecular Pharmacology, Blavatnik Institute, Harvard Medical School, Boston, MA 02115; 10Computer-Aided Molecular Engineering, Department of Oncology, Ludwig Institute for Cancer Research, University of Lausanne, CH-1005 Lausanne, Switzerland; and 11Department of Pathology, Brigham and Women’s Hospital, and Harvard Medical School, Boston, MA 02115

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Notch pathway signaling is implicated in several human cancers. Ablative activation and mutations of Notch signaling components are linked to tumor initiation, maintenance, and resistance to cancer therapy. Several strategies, such as monoclonal antibodies against Notch ligands and receptors, as well as small-molecule γ-secretase inhibitors (GSIs), have been developed to interfere with Notch receptor activation at proximal points in the pathway. However, the use of drug-like small molecules to target the downstream mediators of Notch signaling, the Notch transcription activation complex, remains largely unexplored. Here, we report the discovery of an orally active small-molecule inhibitor (termed CB-103) of the Notch transcription activation complex. We show that CB-103 inhibits Notch signaling in primary human T cell acute lymphoblastic leukemia and other Notch-dependent human tumor cell lines, and concomitantly induces cell cycle arrest and apoptosis, thereby impairing proliferation, including in GSI-resistant human T cell lines, and concomitantly induces cell cycle arrest and apoptosis, thereby impairing proliferation, including in GSI-resistant human tumor cell lines with chromosomal translocations and rearrangements in Notch genes. CB-103 produces Notch loss-of-function phenotypes in flies and mice and inhibits the growth of human breast cancer and leukemia xenografts, notably without causing the dose-limiting intestinal toxicity associated with other Notch inhibitors. Thus, we describe a pharmacological strategy that interferes with Notch signaling by disrupting the Notch transcription complex and shows therapeutic potential for treating Notch-driven cancers.

Notch | small molecule inhibitor | cancer

Transcription factors (TFs) are key mediators of cellular processes and cell states. In cancer, TFs are commonly deregulated indirectly by aberrant upstream signaling cascades or directly by pathogenic mutations and or translocations. Although in principle an attractive class of therapeutic targets, TFs are largely considered undruggable due to the absence of surface pockets amenable to effective targeting with small molecules. Thus, most currently available targeted cancer therapeutics aim at inhibiting oncogenic signaling pathways at the most proximal part of the cascade, either through antibodies against ligands or surface receptors, or using small molecules that inhibit the enzymatic activities of receptor-associated kinases.

The Notch signaling cascade is one example of a pathway that has emerged as a rational therapeutic target in several cancers. In the adult, Notch signaling in progenitor and stem cells regulates tissue homeostasis, self-renewal, and differentiation in several organs and cell types, including the intestine, vasculature, and hematopoietic system (1).

Notch signaling is initiated through the interaction of a receptor and ligand on neighboring cells. This event results in sequential proteolytic cleavages of the receptor mediated by metalloproteases of the ADAM family and the γ-secretase multiprotein complex that liberate the Notch intracellular domain (NICD). NICD subsequently traffics to the nucleus, binds the TF RBPJ, and recruits other coactivators, such as mastermind proteins (MAML1-3) and p300, forming a transcription activation complex that initiates the expression of downstream target genes (2).

Next-generation sequencing of cancer genomes has identified numerous oncogenic gain-of-function mutations in NOTCH1 or NOTCH2 in B and T cell leukemias and lymphomas (3–5) and solid cancers, such as adenoid cystic carcinoma (6) and breast carcinoma (7, 8). These mutated or truncated genes encode proteins that are processed to NICD constitutively or have increased stability in their active forms, increasing the expression

Significance

The Notch signaling cascade is deregulated by oncogenic lesions in human cancers and has therefore become an attractive therapeutic target. Inhibitory monoclonal antibodies and small-molecule γ-secretase inhibitors have been developed to target the pathway at the most proximal point of the cascade. Major hurdles to the therapeutic application of these Notch inhibitors have been prevalent dose-limiting toxicities in the intestine. Here we report identification and preclinical validation of a small molecule (CB-103) that inhibits the pathway at the level of the Notch transcription complex without causing intestinal toxicity. Its properties and mechanism of action provide CB-103 with a more favorable therapeutic index than other types of Notch targeting agents, a feature that is currently being tested in cancer patients.


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Data deposition: The SLAM-seq data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE148228).

1To whom correspondence may be addressed. Email: freddy.radtke@epfl.ch.

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of target genes that deregulate cell growth and survival. Several strategies, such as monoclonal antibodies (MAbs) against Notch ligands (9–11) and receptors (12, 13), and small-molecule γ-secretase inhibitors (GSIs) (14, 15), have been developed to block Notch receptor activation at proximal points in the pathway. Whereas MAbs have the advantage of specifically inhibiting individual ligands or receptors, GSIs are pan-Notch inhibitors that block signaling through all four Notch receptors (16). GSIs were originally developed for treating Alzheimer disease because they inhibit the cleavage of amyloid precursor protein. In addition, they also block the proteolytic cleavage step (S3 cleavage) that generates NICD, leading to their widespread experimental use as Notch inhibitors (1, 14). However, GSIs also block the processing of more than 90 other substrates, which may complicate the interpretation of results produced by GSIs (16). Although both MAbs and GSIs have shown beneficial effects in preclinical Notch-driven tumor models and clinical studies (12, 17–21), none of these Notch inhibitors have been clinically approved, largely due to on-target dose-limiting toxicities of the intestinal epithelium (22, 23). Treatment of patients with GSIs is frequently associated with diarrhea, vomiting, and nausea, which may occur with severe (24, 25). To avoid this toxicity, clinical trials in Notch-driven cancers have relied on intermittent dosing of GSIs (14). However, the question remains as to whether intermittent dosing strategies sustain Notch inhibition long enough to achieve therapeutic efficacy.

There have also been attempts to target the pathway downstream of the γ-secretase–mediated activation of Notch receptors. One is based on the finding that truncated forms of MAML1 that bind the RBPJ–NICD complex but lack the ability to recruit other coactivators function in a dominant-negative manner (26–28). Based on this concept, Bradner and colleagues (29) synthesized a stapled peptide named SAHM1 (stapled α-helical peptide derived from MAML1) designed to mimic dominant-negative forms of MAML1. However, developing drug-like stapled peptides as therapeutics remains challenging due to manufacturing, stability, and pharmacokinetic issues. Another approach utilized screens to identify the small molecule Mastemind recruitment-1 (IMR-1), which is also proposed to have dominant-negative MAML-like activity (30). Finally, a recent report describes the identification of a small molecule that blocks the interaction between RBPJ and SARP, a protein that forms a corepressor complex with RBPJ (31). However, this approach does not inhibit NOTCH signaling, but rather leads to derepression of NOTCH target genes (31). Although all of these Notch TF complex-modulating compounds show inhibitory activities in cellular assays, it remains to be determined whether these inhibitors possess drug-like properties, as none of these compounds have been tested in clinical trials.

Here, we report the discovery and preclinical validation of an orally active small molecule [6-4-(tert-butyloxyphenoxy)pyridine-3-amine, termed CB-103] that interferes with the function of the Notch transcription complex. CB-103 induces loss-of-function phenotypes in flies and mice and has antitumor activity in xenograph models of Notch-addicted human leukemia and carcinoma without causing gut toxicity. CB-103 possesses excellent drug-like properties and is currently being evaluated in a phase I clinical trial in cancer patients.

Results

Identification of CB-103 as a Notch Inhibitor. Seeking to discover small-molecule inhibitors of Notch signaling, we developed a cell-based coculture assay amenable to high-throughput screening of chemical compound libraries. This screen utilized a coculture system consisting of “signal-receiving” HeLa cells expressing Notch1 in combination with a Notch-responsive luciferase reporter, and “signal-sending” HeLa cells expressing the Notch ligand Delta-like 4 (DL4) (Fig. 1A and B).

A screen of 67,253 compounds from commercially available libraries identified 341 compounds with Notch inhibitory activity. Computer-aided self-organizing mapping programs that cluster compounds based on structural similarities allowed reduction of the number of hits to 98, of which 33 were validated by a secondary screen in the same coculture assay. These compounds were then assayed for their ability to block signaling driven by a dominant active form of the Notch1 receptor (N1-ICD) (Fig. 1C). Importantly, this approach enables identification of compounds that act downstream of the γ-secretase–mediated S3 cleavage event. Employing this strategy, we identified the compound CB-103 (Fig. 1D). To assess whether CB-103 specifically inhibits Notch1 or is also active against other Notch receptors, we tested the ability of CB-103 to inhibit Notch2-, Notch3-, and Notch4-mediated signaling using the in vitro coculture assay. CB-103 inhibited Notch signaling mediated by each of the receptors tested in a dose-dependent manner (Fig. 1E and F). As was observed for N1-ICD (Fig. 1G), CB-103 was also able to block the activity of the dominant active forms of Notch2, Notch3, and Notch4 (N2-ICD, N3-ICD, and N4-ICD, respectively) (Fig. 1H). Overall, CB-103 inhibited both ligand-dependent and ligand-independent Notch activation in cell-based assays, with IC50 values ranging from 0.9 to 3.9 μM (Fig. 1 F and H); CB-103 did not inhibit Wnt or Hedgehog signaling using reporter assays (SI Appendix, Fig. S1). Importantly, this profile of the above compounds, CB-103 acts to prevent Notch-mediated transactivation and thus sought to substantiate this prediction. Experiments using a N1-ICD–GFP fusion protein indicated that CB-103 does not prevent nuclear translocation of N1-ICD (Fig. 1J), excluding impaired trafficking as a mechanism of action. An alternative possibility is that it interferes with a functional assembly of the transcription complex. Consistent with this, expression of increasing amounts of the cofactor MAML1 counteracted the inhibitory effect of CB-103 (Fig. 1J), suggesting that CB-103 impairs the recruitment and assembly of components of the Notch transcription complex.

CB-103 Inhibits Growth of Notch-Addicted Human T Cell Acute Lymphoblastic Leukemia Cell Lines through Modulation of the Notch Transcription Complex. To further establish CB-103 as a bona fide Notch inhibitor, we tested its ability to directly inhibit the growth of Notch-dependent cancer cells by initially focusing on T cell acute lymphoblastic leukemia (T-ALL). More than 50% of T-ALL patients harbor activating NOTCH1 mutations resulting in increased Notch signaling (3). Treatment of the NOTCH1-mutated human T-ALL cell line RPMI-8402 and the NOTCH3-driven human TALL-1 cell line with CB-103 or a GSI resulted in down-regulation of the Notch target genes HES1, MYC, and DTX1 (SI Appendix, Fig. S2A and B), as well as down-regulation of NOTCH1 (SI Appendix, Fig. S2C). Interestingly, protein levels of N1-ICD were unaffected by short-term (6 h) treatment with CB-103 (SI Appendix, Fig. S2C), but were significantly reduced at later time points (24 and 48 h) (SI Appendix, Fig. S2D and E). In contrast, protein levels of RBPJ were unaffected (SI Appendix, Fig. S2E). Simultaneous treatment of RPMI-8402 cells with CB-103 and a proteasome inhibitor did not restore N1-ICD levels, suggesting that CB-103 does not impact N1-ICD protein levels by enhancing proteasomal degradation (SI Appendix, Fig. S2F). As NOTCH1 exhibits positive autoregulation in T-ALL cells, these results are consistent with a fall in N1-ICD levels due to inhibition of NOTCH1 transcription. In addition, CB-103 induced profound cell growth inhibition in both RPMI-8402 and T-ALL1 cells (SI Appendix, Fig. S2G and H). In contrast, growth of the Notch-independent HeLa cell line was unaffected by either CB-103 or GSI treatment (SI Appendix, Fig. S2J). Global gene-expression analysis of CB-103-treated NOTCH1-mutated T-ALL cell lines KOPT-K1 and HBP-ALL further confirmed down-regulation of N1-ICD–driven growth-promoting...
genes, including MYC, the main oncogenic driver directly regulated by Notch in T-ALL (32, 33) (SI Appendix, Fig. S3).

To gain insight into the molecular mechanism underlying CB-103 inhibition of Notch-mediated transcription, we generated RPMI-8402 T-ALL cell lines with reduced sensitivity to CB-103 and then investigated a possible mechanism of insensitivity (mutation) by exome sequencing, motivated by the hypothesis that drug insensitivity could occur through gene mutations affecting the drug’s targets (34). Exome sequencing of CB-103 insensitive RPMI-8402 T-ALL cells identified a G193R mutation in CB-103.
within the BTD domain of RBPJ. Importantly, engineered expression of a V5-tagged RBPJ<sup>193R</sup> mutant gene in parental RPMI-8402 cells shifted the IC<sub>50</sub> for CB-103 from 2.6 μM to >100 μM, whereas expression of V5-tagged WT RBPJ had minimal effects, indicating that this specific single amino acid change is sufficient to confer insensitivity to CB-103 treatment (Fig. 2).

Next, we performed computational docking studies. CB-103 was docked on the NOTCH1 transcription complex/HES1 promoter DNA system to determine a possible binding mode on the native structure (35). Among the calculated binding modes, one confirmed the BTD domain of RBPJ as possible binding site for CB-103 and identified several key RBPJ amino acid residues (Fig. 2A). Expression of engineered forms of RBPJ-bearing mutations in these residues—specifically V5-tagged RBPJ<sup>F196A</sup>, V5-tagged RBPJ<sup>L245A</sup> or V5-tagged RBPJ<sup>L248A</sup>, but a control mutant V5-tagged RBPJ<sup>G194R</sup>—in parental RPMI-8402 cells conferred resistance to CB-103 (Fig. 2B and C and SI Appendix, Fig. S4). Thus, combined docking and mutational analysis support the binding of CB-103 to this pocket in the BTD domain. Notably, this pocket is also important for binding of the N1-ICD RAM domain to RBPJ, providing an explanation for how CB-103 impairs the formation and activity of the NOTCH1 transcription complex.

In further support of this model, CB-103 also interfered with recovery of N1-ICD in immunoprecipitates prepared from RPMI-8402 cells expressing V5-WT-RBPJ, but not from cells expressing V5-RBPJ<sup>G193R</sup> mutant (Fig. 3A). Furthermore, CB-103 reduced the occupancy of RBPJ and N1-ICD on genomic Notch-response elements associated with the Notch target genes HES1, DTX1, and MYC in RPMI-8402 cells expressing V5-WT-RBPJ but not in cells expressing the V5-RBPJ<sup>G193R</sup> mutant (Fig. 3C). Taken together, these results strongly suggest that CB-103 inhibits Notch-mediated transcription by interfering with assembly of the Notch transcription complex.

The majority of Notch/RBPJ binding sites that mediate acute changes in gene expression are found in enhancers (36). These genomic response elements are of two distinct types, one containing monomeric RBPJ sites and the second dimeric head-to-head RBPJ sites separated by 15 to 17 base pairs, an element called a sequence-pair site (SPS) that supports loading of dimeric Notch TF complexes. SPS-mediated Notch signaling

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**Fig. 2.** Single amino acid mutations within the BTD domain of RBPJ cause unresponsiveness to CB-103 in RPMI-8402 cells. (A) Experimental structure of the NOTCH1 transcription complex on the HES1 promoter DNA sequence (PDB ID code 3V79) (64). RBPJ (orange), domains are shown in ribbon representation. HES1 backbone is shown in ribbon representation, with nucleotides displayed as tubes. Green arrows indicate predicted amino acids important for binding of CB-103 within the BTD domain of RBPJ. (B) Graph shows dose–response curves of CB-103–treated parental, RBPJ<sup>wt</sup>–V5- and RBPJ<sup>G193R</sup>–V5-, RBPJ<sup>L245A</sup>–V5-, RBPJ<sup>L248A</sup>–V5-, RBPJ<sup>F196A</sup>–V5-, and RBPJ<sup>G194R</sup>–V5-expressing RPMI-8402 cells. Cells were treated with CB-103 for 3 d. IC<sub>50</sub> values for respective cell lines are indicated. (C) Bar graphs show percentage of apoptotic cells of parental, RBPJ<sup>wt</sup>–V5- and RBPJ<sup>G193R</sup>–V5-, RBPJ<sup>L245A</sup>–V5-, RBPJ<sup>L248A</sup>–V5-, RBPJ<sup>F196A</sup>–V5-, and RBPJ<sup>G194R</sup>–V5-expressing RPMI-8402 cells treated with DMSO or CB-103 (10 μM) for 72 h. Statistical analysis was performed using two-tailed t test (**P < 0.0005, ***P < 0.007; ns, not significant).
is important for T cell maturation and leukemic transformation but dispensable for T cell fate specification in mice (37, 38). To determine whether CB-103 preferentially inhibited elements containing SPSs versus head-to-tail–oriented RBPJ binding sites, we performed luciferase reporter gene assays. Although SPS-driven reporters elicited a much stronger signal compared to head-to-tail–oriented RBPJ-driven promoters, both were equally sensitive to CB-103. Thus, CB-103 inhibits both monomeric and dimeric Notch1 TF complexes, which agrees with the proposed mode of action of CB-103 (SI Appendix, Fig. S5).

Next, we performed kinetic gene-expression analysis using SLAM-seq [thio(SH)-linked alkylation for the metabolic sequencing of RNA] on vehicle and CB-103–treated RPMI-8402 T-ALL cells to investigate potential differences in the CB-103 sensitivity of promoter- and enhancer-driven Notch target genes. Pathway analysis from the Hallmark collection revealed E2F targets, MYC targets, and PI3K AKT MTOR signaling as being the most rapidly down-regulated pathways. Interestingly, the Notch target genes DTX1, HES1, NOTCH3, and NOTCH1, which are regulated by response elements found in promoters or intragenic enhancers, were down-regulated faster than target genes that are regulated by long-distance enhancers—such as MYC, GIMAP1, -5, -6, and -8—suggesting that different Notch target genes may have varying sensitivities and or kinetics of response to CB-103 (SI Appendix, Fig. S6).

In previous studies, the stapled peptide and small molecules SAHM1 and IMR-1 were also claimed to target the Notch transcription complex (29, 30); we therefore compared their activities with CB-103 in reporter gene assays and in Notch-driven T-ALL cells. The activity of SAHM1 in reporter gene assays was indistinguishable from unstacked control peptide; and although SAHM1 was cytotoxic, unlike CB-103, it did not down-regulate MYC expression in RPMI-8402 T-ALL cells (SI Appendix, Fig. S7). In the same assays, we failed to identify any effect of IMR-1 on MYC levels or RPMI-8402 cell growth at doses up to 10 μM (SI Appendix, Fig. S7). Thus, CB-103 acts by a markedly different mechanism than SAHM1 and IMR-1 with respect to the expected activities of direct Notch transcription complex.

**CB-103 Function In Vivo Recapitulates Genetic Notch Loss-of-Function Phenotypes without Causing Gut Toxicity.** Prior to investigating the in vivo activity of CB-103, we profiled the compound to determine its drug-like and ADME/PK (absorption, distribution, metabolism, excretion, and pharmacokinetic) properties (SI Appendix, Fig. S8). Then we assessed CB-103’s ability to affect a variety of Notch-dependent cellular processes. First, we studied the effect of CB-103 on the development of mechanosensory organs in Drosophila, which consist of four distinct lineages (socket, shaft, sheath, and neuron) derived from a single sensory organ precursor cell (39). Notch signaling loss induces aberrant sensory organ lineages, including lineages composed of two neurons and no sheath cells, indicative of sheath-to-neuron transformation (39). Treatment of developing sensory organs with CB-103 resulted in increased numbers of neurons, similar to the effect produced by GSI (DAPT), demonstrating that CB-103 inhibits Notch-dependent cell fate specification in flies (SI Appendix, Fig. S9).

We extended these findings to mammals and investigated how CB-103 affects five Notch-dependent phenotypes in mice: 1) Development of splenic marginal zone B (MZB) cells, 2) thymic T cell development, 3) generation of Eosin+ dendritic cells, 4) sprouting of endothelial cells, and 5) induction of goblet cell differentiation in the small intestine. Each of these processes strictly require Notch signaling (40–46). As expected, treatment with CB-103 (SI Appendix, Fig. S10) resulted in a dramatic, reversible reduction of B220+CD21hiCD23low MZB cells (SI Appendix, Fig. S10).
mimicking phenotypes caused by loss of Notch2 (38) or Dll1 (39). Osmotic pump-mediated delivery to maintain sustained exposure to CB-103 resulted in a concentration-dependent reduction of MZB cells and revealed that a level of 700 ng/ml (2.8 μM) is sufficient to inhibit Notch signaling (SI Appendix, Fig. S10 C and D). Similarly, we observed impaired thymic T cell development (SI Appendix, Fig. S11A), reduced numbers of Esam<sup>+</sup> CD11c<sup>+</sup> CD8<sup>+</sup> dendritic cells (SI Appendix, Fig. S11B), and increased endothelial cell sprouting in CB-103-treated WT mice (SI Appendix, Fig. S12).

In notable contrast, CB-103 treatment did not produce the expected intestinal toxicity. Genetic deletion of genes encoding Notch1 and Notch2 receptors or Dll1 and Dll4 ligands or Rhbp, as well as sustained treatment with potent GSIs, result in goblet cell metaplasia and reduced proliferation of crypt progenitor cells (40, 41, 47). Goblet cell metaplasia-associated intestinal toxicity is one of the main dose-limiting “adverse events” in clinical trials using Notch-targeting agents, such as GSI or antagonistic Notch receptor antibodies (1). We treated mice with vehicle control, CB-103, or the GSI LY3039478. Mice treated with vehicle control or CB-103 were analyzed after 1 wk and 4 wk of treatment, while mice treated with LY3039478 were analyzed after 1 wk due to treatment-related morbidity. Alcian blue and K<sub>d</sub>f<sub>7</sub> staining revealed a dramatic increase in goblet cell numbers and a highly significant reduction in crypt cell proliferation in LY3039478-treated animals. In contrast, CB-103-treated animals showed only a moderate increase in goblet cell numbers and a moderate reduction in K<sub>d</sub>f<sub>7</sub><sup>+</sup> crypt cells (Fig. 4).

To investigate the basis of the milder gut phenotype in CB-103–treated mice, we studied its effects on key downstream target genes. In the small intestine, Notch signaling directly regulates the expression of the stem cell marker gene Otnm4 and members of the Hes family of TFs, which repress the transcriptional master regulator for secretory cells, Atoh1 (48). In situ hybridization studies revealed that the expression of Otnm4 and Hes1 was nearly completely abrogated by CB-103 and LY3039478, thereby demonstrating Notch inhibitory activity of these agents in the gut. In contrast, LY3039478-treated animals had significantly higher levels of Atoh1 transcripts compared to vehicle- and CB-103–treated animals, which showed only a small increase in Atoh1 transcripts relative to vehicle-treated animals (Fig. 4B). To exclude possible confounding influences of variation in absorption or pharmacokinetic properties in vivo, we tested these compounds in intestinal organoid cultures created from WT mice. LY3039478-treated organoids had a very different morphology than CB-103–treated cultures and showed increased goblet cell differentiation and decreased proliferation compared to vehicle- or CB-103–treated organoids (SI Appendix, Fig. S13A). We again observed strong inhibition of the Notch pathway, as indicated by down-regulation of direct Notch target genes Otnm4, Hes1, Hes3, and Hes5 to a similar degree by both CB-103 and LY3039478, whereas Atoh1 transcripts were up-regulated to higher levels in LY3039478-treated organoids (SI Appendix, Fig. S13B). These results show that while both compounds block Notch signaling in the intestine in vivo and in intestinal organoids, goblet cell metaplasia and profound growth inhibition were only observed with LY3039478 treatment, presumably due to the higher expression levels of Atoh1.

**CB-103 Blocks Tumor Growth of GSI-Resistant Cancers.** A potential advantage of a small-molecule inhibitor of the Notch TF complex is the ability to block Notch signaling in tumors that are resistant to Notch-directed MAbs or GSIs by virtue of Notch gene rearrangements that lead to y-secretase independent Notch activation. As a proof-of-principle, we studied via lentiviral expression of constitutively nuclear N1-ICD whether CB-103 could inhibit growth of Notch-dependent human DND-41 T-ALL cells rendered resistant to agents (such as GSI) that act upstream of the Notch TF complex. As expected, parental DND-41 cells exhibited reduced growth inhibition and Notch signaling following treatment with either LY3039478 or CB-103, whereas only CB-103 elicited growth arrest in DND-41 cells expressing N1-ICD (SI Appendix, Fig. S14A). CB-103 sensitivity was in turn rescued by ectopic expression of MYC (SI Appendix, Fig. S14B), consistent with prior work implicating MYC as a key Notch target gene in T-ALL cells (51).

Next, we investigated the ability of CB-103 to inhibit growth of Notch-dependent cancers that are resistant to MAbs and GSI therapy. We focused on triple negative breast cancer (TNBC), since ∼10% have rearrangements in NOTCH1 and/or NOTCH2 that lead to constitutive, ligand-independent Notch activation (7, 8). As predicted, growth of the GSI-resistant HCC1187 cell line carrying a NOTCH2 rearrangement (7) was inhibited by CB-103 treatment but not by RO4929097, a GSI previously assessed in clinical phase I/II studies (8) (Fig. 5A). We subsequently established a stable luciferase reporter line to determine the ability of CB-103 to inhibit growth of HCC1187 following xenotransplantation. CB-103–treated animals showed remarkable growth inhibition and reduced tumor burden compared to vehicle-treated animals (Fig. 5B). CCND1, a known Notch target in breast cancer (52, 53), was down-regulated in tumors from CB-103–treated animals compared to controls (Fig. 5C). Moreover, CB-103–mediated tumor growth inhibition was accompanied by reduced K<sub>d</sub>f<sub>7</sub> and increased Caspase 3 staining, while CD31 staining was similar in vehicle- and CB-103–treated animals (SI Appendix, Fig. S15). Our data provide proof-of-concept that CB-103 can inhibit growth of “Notch-addicted” cancer cells expressing mutated forms of Notch that cannot be targeted with agents that act upstream of the Notch TF complex. Furthermore, patient-derived xenotransplantation (PDX) experiments using a NOTCH1<sup>+</sup> oxaloplatin-resistant colorectal cancer sample (54) revealed that CB-103 resensitizes this tumor to oxaloplatin treatment in vivo (Fig. 5D).

To extend these findings to primary human cancers, we investigated the ability of CB-103 to inhibit the growth of 19 primary T-ALLs, the human tumor with the highest frequency of Notch gain-of-function mutations, in a coculture model (47). Dose–response profiles indicated that CB-103 induced growth inhibition in ∼50% of the cases tested with IC<sub>50</sub> values in the submicromolar range (Fig. 5E). Importantly, the ability of CB-103 to reduce tumor growth correlated strictly with Notch activation status, as only tumors containing N1-ICD responded to CB-103 treatment. Furthermore, growth inhibition induced by CB-103 was associated with an increased level of N1-ICD pretreatment and decreased N1-ICD levels posttreatment, but did not strictly correlate with the mutation status of NOTCH1 or FBXW7 (Fig. 5F). These results indicate that CB-103 selectively inhibits the growth of T-ALLs with ongoing NOTCH1 activation and supports prior studies suggesting that the level of N1-ICD predicts tumor response to Notch pathway inhibitors (44). In line with these ex vivo results, CB-103 prolonged the survival of mice bearing a NOTCH1-mutated T-ALL PDX model compared to vehicle treatment (Fig. 5 G, Left), an antitumor effect that was also associated with decreased N1-ICD levels (Fig. 5 G, Right). A second independent N1-ICD<sup>+</sup> T-ALL PDX model also showed significantly reduced tumor burden in mice with either high or low tumor burden (>20% and <2% leukemic blasts in the peripheral blood) at treatment initiation, as indicated by percentages of circulating huCD45<sup>+</sup>CD7<sup>+</sup> T-ALL blasts after CB103 treatment (Fig. 5H).

**Discussion**

In the last decade unbiased next-generation sequencing efforts of human cancer specimens have identified activating genetic aberrations in NOTCH1 genes in a broad spectrum of cancers, including T-ALL, adenoid cystic carcinoma, chronic lymphocytic leukemia, MZB cell lymphoma, and breast cancer (3, 6, 7, 55). Moreover, many preclinical studies have implicated Notch signaling in almost all hallmarks of cancer, highlighting why this signaling pathway is an intriguing but complex therapeutic target (56).
Herein we describe a cell-based high-throughput screen that led to the identification and characterization of a small-molecule inhibitor (CB-103) of the Notch cascade. CB-103 is a pan Notch inhibitor as it can block Notch-mediated signaling of all four Notch receptors (Fig. 1), similar to GSIs. Commonly used GSIs block Notch signaling by inhibiting the proteolytic activity of the γ-secretase multiprotein complex, which cleave Notch receptors at the S3 site within the transmembrane domain. In contrast to GSIs, CB-103 evidently inhibits the pathway at the most downstream level—the Notch transcriptional complex—based on its ability to block GSI-insensitive dominant active forms of NICD. Further evidence comes from the generation of a T-ALL cell line that is resistant to CB-103. The rationale of this experimental design was that drug insensitivity can be afforded through mutations that circumvent the inhibitory activity by the drug, mutations that have the potential to reveal mechanistic insights both into the actions of the drug and its target (34). In the past this approach has been used successfully to identify drug targets for BI 2536, a Polo-like kinase1 inhibitor (50), as well as for the proteasome inhibitor Bortezomib, which is clinically used to treat multiple myeloma and mantle cell lymphoma (34, 57). Using proteasome inhibitor Bortezomib, which is clinically used to treat multiple myeloma and mantle cell lymphoma (34, 57). Using proteasome inhibitor Bortezomib, which is clinically used to treat multiple myeloma and mantle cell lymphoma (34, 57). Using proteasome inhibitor Bortezomib, which is clinically used to treat multiple myeloma and mantle cell lymphoma (34, 57). Using proteasome inhibitor Bortezomib, which is clinically used to treat multiple myeloma and mantle cell lymphoma (34, 57).

target genes, such as HES1, DTX1, and MYC (Fig. 3). In addition, computational docking studies identified the BTD domain of RBPJ as potential binding pocket for CB-103, and accurately predicted amino acids residues in RBPJ that when mutated confer resistance to CB-103. Taken together, the computational docking studies, mutational analysis, as well as pulldown and ChIP experiments are in agreement with CB-103 acting as a direct inhibitor of the Notch transcription complex.

Oncogenic Notch signaling can be triggered by a variety of mutations, including single nucleotide substitutions, small insertion/deletion mutations, and rearrangements of Notch genes. One advantage of CB-103 over currently available Notch inhibitors is that it is active against tumor cells bearing any of these types of mutations, which is not uniformly true of other Notch inhibitors. Mutated Notch receptors found in tumors with Notch gene rearrangements lack ectodomains and therefore do not rely on ligand for activation and cannot be targeted with blocking antibodies. Furthermore, ~5% of TNBCs and 52% of glomus tumors have gene rearrangements in NOTCH2 (7, 8, 58) in which N2-ICD nuclear access is not γ-secretase-dependent, as translational initiation in the aberrant NOTCH2 transcripts lies C terminal of the S3 cleavage site (7). We performed proof-of-concept experiments (Fig. 5 and SI Appendix, Fig. S14) using either engineered human T-ALL cell lines expressing N1-ICD or the TNBC cell line HCC1187, which has a NOTCH2 gene rearrangement. Notably, CB-103 inhibited growth of both cell lines in vitro as well as the growth of HCC1187 xenotransplants, whereas GSIs were inactive.

Previous studies also identified compounds (SAHM1, IMR-1, and RIN1) that were claimed to target the Notch transcription of
CB-103 impedes growth of NOTCH+ cancer cell lines and primary human T-ALL. (A) Growth kinetics of HCC-1187 cells treated with DMSO, GSI (RO4929097), and CB-103 (10 μM each) for 6 d. (B) Luciferase-expressing HCC-1187 cells were subcutaneously transplanted into NOD/SCIDγc−/− (NSG) mice and treated with either vehicle (n = 6) or CB-103 (n = 6) for 15 d (2× a day). Bioluminescence was measured 30 d posttransplantation. HCC-1187 tumor volume (mm³) measured over time in xenotransplanted mice treated with either vehicle or CB-103 (25 mg/kg) administrated twice a day (n = 6 for each group) is shown. (C) Representative H&E staining (Left) and immunostaining for Cyclin D1 (Right) of tumors harvested from vehicle- and CB-103–treated animals is shown. (D) Oxaliplatin-resistant M43 colorectal cancer cells were transplanted into NSG mice and subsequently treated with either vehicle (n = 18), CB-103 (n = 18), oxaliplatin, or oxaliplatin and CB-103 for 12 d (25 mg/kg 1× a day) and tumor growth fold-change was monitored over time (Left) and at end stage of the experiment (Right). One-way ANOVA, Tukey’s multiple comparisons test *P < 0.05; ***P < 0.0003; ****P < 0.0001; ns, not significant. (E) Response to CB-103 of T-ALL cells in vitro. Patient-derived xenografts (n = 20) were maintained on human mesenchymal stromal cells and incubated with 0.01, 0.1, 1, 10, or 25 μM CB-103 for 7 d. Graph represents IC₅₀ values of each patient sample treated with CB-103. Blue circles represent patient samples positive for the presence N1-ICD, red circles represent patient samples negative for N1-ICD. (F) Immunoblots underneath show N1-ICD levels decrease upon treatment with CB-103. N1-ICD detection in indicated patient samples (numbered) on Western blot after 72-h exposure to DMSO or CB-103 is shown. Mutation status for NOTCH1 and FBXW7 are indicated. (G) Event-free survival analysis after treatment of leukemia xenografts of the T-ALL corresponding to case 1. Kaplan–Meier survival curve is shown for xenografted NSG mice. Treatment with vehicle or CB-103 as indicated when 10% of T-ALL cells were detected in peripheral blood by flow cytometry. Log-rank (Mantel–Cox) test and P value as indicated. Bar graph shows detection levels of N1-ICD in T-ALL cells after in vivo treatment with CB-103. The mean ratio (± SEM) of N1-ICD (Val-1744) determined in vehicle and CB-103–treated animals (two per condition). A representative Western blot example for N1-ICD of treated animals is shown. (H) Bar graphs show absolute numbers of huCD45+CD7+ T-ALL cells after treatment. Animals with high tumor burden: n = 5 for vehicle control and n = 3 for CB-103–treated animals; animals with low tumor burden: n = 5 for both vehicle and CB-103 treated animals. Statistical analysis: Student’s t test test, *P < 0.05.
complex (29–31). However, in reassessing the activity of SAHM1, we were unable to confirm any “on-Notch” inhibitory effects in reporter gene assays or T-ALL cells (SI Appendix, Fig. S7). IMR-1 is a small chemical compound that was identified based on the strategy of inhibiting MAML1 recruitment to the Notch transcription complex (30). A side-by-side comparison of CB-103, the GSI LY3039478, SAHM1, and IMR-1 for their ability to block growth of a Notch-driven human T-ALL cell line showed that IMR-1 had no effect on Notch target gene expression or cell growth at doses up to 10 μM, whereas CB-103 and GSI were active (SI Appendix, Fig. S7). IMR-1 evidently can inhibit Notch signaling at higher concentrations, as previously reported (30, 59, 60). RIN1 is another small molecule that was recently reported to modulate the Notch transcription complex (31). This small chemical compound was identified in a reporter-based cell culture assay aimed at identifying inhibitors of interactions between RBPJ and SHARP, which is a component of a transcriptional repressor complex that binds RBPJ in the absence of NICD. In line with this possibility, RIN1-treated cells up-regulated Notch target genes, mimicking the effects of RBPJ knockdown. The activity of RIN1 has yet to be assessed, in regard to therapeutic efficacy and intestinal toxicity (31), and comparative evaluation with CB-103 warrants future consideration.

One of the major dose-limiting toxicities and hurdles to the therapeutic application of the pan-Notch inhibitor has been intestinal toxicity (22, 23). Genetic studies in mice show that Notch acts as a stem and progenitor cell gate keeper and is important for secretory versus absorptive cell fate differentiation. Conditional, gut-specific inactivation of Notch1 and -2, the ligands Dll1 and Dll4, and Rbpj result in the loss of proliferative crypt progenitors and conversion into postmitotic goblet cells (40, 41, 47). A similar phenotype has been observed in mice with simultaneous gut-specific inactivation of the Notch target genes Hes1, Hes3, and Hes5, indicating Notch signaling regulates intestinal homeostasis at least in part through Hes genes (49). Conversely, transgenic Notch gain-of-function experiments cause a reciprocal phenotype consisting of a block in secretory cell differentiation and an expansion of immature crypt progenitors (61). The gut phenotype created by knockout of Notch pathway components is also observed in rodents treated with potent GSIs, such as dibenzazepine (47), suggesting that the gut toxicity caused by GSI is mostly driven by Notch inhibition (16). In this context, it is interesting to note that four different γ-secretase complexes exist and that most available GSIs block all complexes, which is likely to account for the intestinal toxicities in GSI-treated animals or patients. A recent report showed that selective pharmacological inhibition of one (presenilin-1) of the four (PSEN) γ-secretase subclasses is effective in reducing the leukemic burden of PSEN-1 expressing T-ALL cells in xenotransplantation assays without causing intestinal toxicity (62). Thus, selective inhibition of the γ-secretase complex might be a potential therapeutic strategy for safely targeting Notch-driven tumors, provided Notch cleavage is mostly mediated by specific PSEN subclasses.

These effects of GSI involve alterations in the TF Atoh1 (also known as Math1), which is a master regulator of the secretory lineage (50, 63). Conditional gut-specific inactivation of Atoh1 results in loss of all secretory cells and in the context of GSI-mediated Notch inhibition has been shown to be essential for goblet cell fate conversion (63). Unexpectedly, in vivo administration of CB-103 to mice did not lead to the anticipated goblet cell metaplasia phenotype, which was observed in GSI-treated animals. Direct Notch target genes, such as Olfm4 and Hes1, were both down-regulated by CB-103 and GSI, indicating that CB-103 reached the target tissue. In contrast, Atoh1 transcripts were significantly higher in GSI-treated animals (Fig. 4). This distinction was confirmed in intestinal organoid cultures, excluding the possibility that this result is a consequence of variation in absorption or pharmacokinetic properties in vivo. LY3039478-treated organoids displayed a very different morphology than CB-103–treated cultures, showing increased goblet cell differentiation and decreased proliferation compared to vehicle- or CB-103–treated organoids (SI Appendix, Fig. S13). The mechanisms underlying this distinction are currently unknown. Several possibilities can be considered. First, CB-103 inhibits protein–protein interaction and might therefore produce more incomplete Notch inhibition than a potent GSI. A second possibility, which is not mutually exclusive, is that CB-103 may inhibit only a subset of RBPJ complexes, leading to variation in the responsiveness of different Notch target genes. Nevertheless, in Notch-driven tumors, where the levels of signaling are well above that of normal tissues due to activating mutations, CB-103 is evidently potent enough to produce responses in preclinical models. Further work is necessary to parse out these distinctions.

In summary, our discovery and characterization of CB-103 makes a compelling case that small-molecule inhibitors can be developed and used to block TF complexes, which are downstream of many aberrant signaling cascades, but have been historically intransigent to therapeutic targeting. CB-103 interferes with the Notch TF complex, and may thereby convey a more favorable therapeutic window than previous Notch-targeting agents. Motivated by this knowledge and by its pharmacological characteristics, CB-103 is currently being evaluated in phase I/II clinical trials (https://clinicaltrials.gov/ct2/show/NCT03422679).

Materials and Methods
Details of materials and methods are provided in SI Appendix, including sources of constructs, compounds, cell lines and cell culture conditions (ex vivo, in vitro), luciferase reporter assay, assays of cell cycle, proliferation, cell viability, stable transformants, and animal studies. Details of microscopy, image processing and analysis, FACS and computational docking studies are described in SI Appendix. Methods for Western blot, immunoprecipitation and ChIP, qRT-PCR, in situ hybridization, histology and immunostaining, RNA sequencing and SLAM-seq, and bioinformatic and statistical analyses are also provided in SI Appendix.

Ethics Statement. All animal work was carried out in accordance with Swiss national guidelines. This study was reviewed and approved by the cantonal veterinary service, Vaud.

Data Availability Statement. All data generated in this study are included in the paper and SI Appendix. The SLAM-seq data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE148228).

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