

**Review Article****Therapeutic Targeting of Notch Signaling Pathway in Hematological Malignancies**Claudia Sorrentino<sup>1</sup>, Antonio Cuneo<sup>1,2</sup> and Giovanni Roti<sup>1</sup>.<sup>1</sup> University of Parma, Department of Medicine and Surgery, Parma, 43126, Italy.<sup>2</sup> University of Ferrara, Department of Medical Sciences, Ferrara, 44121, Italy.**Competing interests:** The authors have declared that no competing interests exist.

**Abstract.** The Notch pathway plays a key role in several processes, including stem-cell self-renewal, proliferation, and cell differentiation. Several studies identified recurrent mutations in hematological malignancies making Notch one of the most desirable targets in leukemia and lymphoma. The Notch signaling mediates resistance to therapy and controls cancer stem cells supporting the development of on-target therapeutic strategies to improve patients' outcome. In this brief review, we outline the therapeutic potential of targeting Notch pathway in T-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, and mantle cell lymphoma.

**Keywords:** NOTCH1, Targeted therapy, T-cell acute lymphoblastic leukemia, Chronic lymphocytic leukemia, Mantle cell lymphoma.**Citation:** Sorrentino C., Cuneo A., Roti G. Therapeutic targeting of notch signaling pathway in hematological malignancies. *Mediterr J Hematol Infect Dis* 2019, 11(1): e2019037, DOI: <http://dx.doi.org/10.4084/MJHID.2019.037>**Published:** July 1, 2019**Received:** March 3, 2019**Accepted:** May 18, 2019This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by-nc/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.Correspondence to: Giovanni Roti. University of Parma, Department of Medicine and Surgery, Parma, 43126, Italy. E-mail: [giovanni.roti@unipr.it](mailto:giovanni.roti@unipr.it)

**Introduction.** Notch pathway comprises a family of single-pass transmembrane receptors, their ligands, and coactivators that regulate evolutionarily conserved signaling that controls development and tissue homeostasis<sup>1,2</sup> in all metazoan organisms. Mammalian NOTCH receptors (NOTCH 1-4) are pre-processed during maturation by a furin-like protease (S1), leading to the formation of two, non-covalently associated subunits. In non-malignant cells, canonical Notch signaling is initiated by cell-to-cell contact of the Notch extracellular domain (NECD) to a ligand of the Delta-like (DLL1, DLL3, DLL4) and Jagged family (JAG1, JAG2), expressed on the cellular surface of the neighboring cell. This receptor-ligand interaction mediates a sequence of two proteolytic cleavages in the Notch transmembrane subunit. The first, resolved by ADAM-10 or ADAM-17 metalloproteases, occurs within a juxtamembrane negative regulatory region (NRR) at a site that is protected in the inactive state (S2).<sup>3-5</sup> This cleavage generates a trans-membrane intermediate that is the substrate for a secondary cleavage (S3) by the  $\gamma$ -secretase, an event that releases

the intracellular domain of NOTCH (ICN, NICD).<sup>6</sup> ICN moves to the nucleus, complexes with the DNA-binding factor RBPJ, and recruits coactivator of the Mastermind-like (MAML) family. The resulting macromolecules complex activates genes transcription but is usually short-lived because the C-terminal portion of ICN (PEST, peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T)) is recognized by an E3 ubiquitin ligase and degraded.<sup>7</sup>

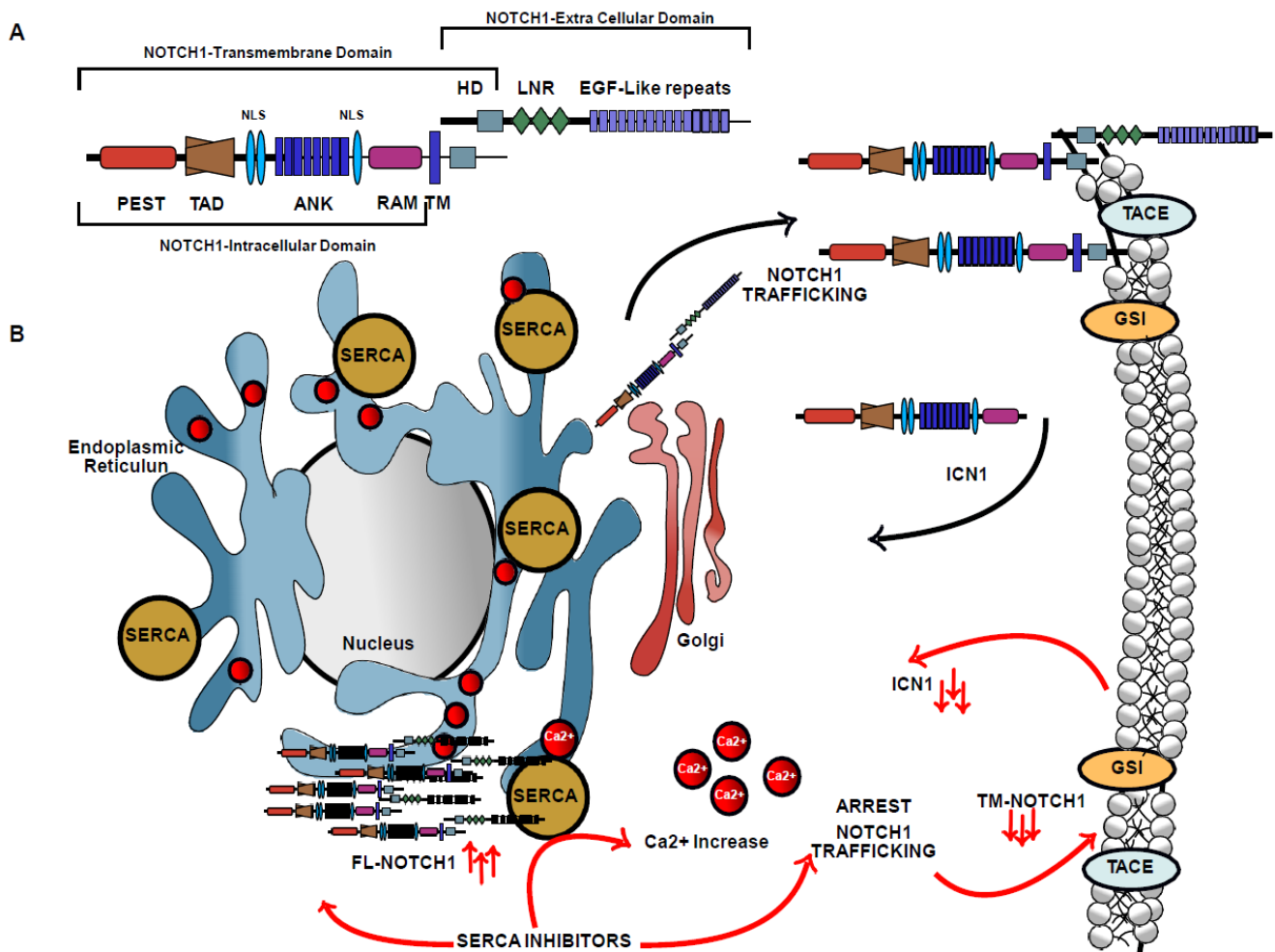
The NOTCH proteins have several functional domains organized in modules. The NECD N-terminal domain is responsible for ligand binding through EGF-like Ca<sup>2+</sup> dependent repeats, followed by three LNR (Lin12/Notch) units. Next to the LNR region lays the juxtamembrane heterodimerization domain (HD), a linker between the extracellular tail and ICN. LNR and HD modules constitute the negative regulatory region (NRR) that prevents ADAM-10/17 cleavage of mammalian Notch in the ligand's absence (**Figure 1A**).<sup>3-5,8</sup>

While oncogenic alterations in the Notch signaling

have been described in almost all human cancers,<sup>3,9</sup> the majority of the recurrent somatic mutations of NOTCH proteins are observed in the *NOTCH1* gene.

The role of NOTCH1 in the pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL), was first investigated in 1991.<sup>1</sup> Ellisen and colleagues described a chromosome translocation, t(7;9)(q34;q34), that juxtaposes the T-cell receptor- $\beta$  to the active form of ICN1 in T-ALL.<sup>10</sup> This fusion creates an oncogenic Notch1 signaling in leukemia cells. Similarly, to the translocation, activating *NOTCH1* mutations generate ligand independent or proteasome resistant ICN1 peptides that sustain T-cell transformation, leukemia growth, or resistance to therapy.<sup>10</sup> In T-ALL, *NOTCH1* mutations cluster in two different but not mutually

exclusive hotspots.<sup>11,12</sup> The first comprises a single amino acid substitution and in-frame insertion in the extracellular NRR. To this class also belongs the rare in-frame insertion in the juxtamembrane extracellular domain (JME). Within the NRR module, most of these mutations occur in the HD domain, and they are defined as type 1A and 1B.<sup>13</sup> Briefly, HD mutations cause ligand-independent Notch conformational changes that constitutively activate ICN1. The second hotspot of *NOTCH1* mutations comprises small insertion/deletion in the exon 34 (PEST domain). These genetic lesions truncate NOTCH1 C-terminal generating a long-lived ICN1 caused by the consequent loss of the “degron” recognition site of the PEST unit.<sup>11,14</sup>



**Figure 1.**

**A)** The schema shows domain organization of NOTCH protein (*NOTCH1* shown). The extracellular domain of NOTCH receptor consists of multiple EGF repeats followed by the NRR (negative regulatory region), which consists of three LNR (Lin-12 and Notch repeats) domains and HD (heterodimerization domain). The intracellular domain of NOTCH receptor consists of a membrane proximal RAM (RBPJ associated molecule) domain, ANK (ankyrin repeats), and a C-terminal TAD (*trans*-activation domain) comprised of three NLS (nuclear localization sequences) and degron-containing PEST (rich in proline, glutamate, serine, and threonine) sequence.

**B)** An overview of Notch1 signaling and proteolytic processing in the presence of SERCA inhibition. NOTCH1 receptor is a cell surface protein. In physiological condition interaction with the Notch ligand, such as JAG1-2 or Dll-4, initiates proteolytic cleavage at the extracellular site by a metalloprotease (TACE) followed by a  $\gamma$ -secretase (GSI) cleavage, resulting in the release of ICN1. ICN1 is then translocated into the nucleus where it interacts with CSL and recruits coactivators to form a transcription-activating complex. In the presence of *NOTCH1* mutations, ICN1 is constitutively active and avoids activation through ligand interaction. Inhibition of the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) leads to alteration in NOTCH1 trafficking causing a loss of NOTCH1 proteins on the surface of the cells and an accumulation of full-length polypeptides on the endoplasmic reticulum/Golgi region. The consequent lack of TACE and GSI substrate causes a reduction in ICN1 level.

Recently, NOTCH1 emerged as one of the most frequently mutated genes (~5-20%) in chronic lymphocytic leukemia (CLL), where it may represent an early driver lesion in a proportion of cases.<sup>15,16</sup> Most of these mutations, ~80%, are a 2-bp deletion in exon 34 that generates a premature stop codon (P2514fs\*4), that truncates the PEST region. Similarly to T-ALL, these mutations cause an over-activation of Notch1 signaling because of the lack of its degradation.<sup>17</sup> Interestingly Kridel and colleagues reported a similar pattern of mutations within the PEST domain in mantle cell lymphoma (MCL).<sup>18,19</sup> Furthermore, 50% of NOTCH1 wild-type CLL cases express ICN1 suggesting that the activation through the canonical Notch signaling is required for leukemia growth in this disease.<sup>20</sup> However, in CLL and MCL, mutations in NOTCH1 are associated with a worse prognosis.<sup>17,21-23</sup> In addition to these observations, Schmitz and colleagues recently described a genetic framework for diffuse large B-cell lymphoma (DLBCL) that may influence the therapeutic response.<sup>24</sup> They identified gain-of-function NOTCH1 mutations (“N1”; these mutations mainly occur in the PEST region) in 19/574 cases of DLBCL. Among these cases, 95% were activated B-cell-like (ABC) diffuse large B-cell lymphoma and no other type of mutation (*BCL6* fusions (B) NOTCH2 (N2), or *SPEN* mutations) co-occurred suggesting that NOTCH1 and NOTCH2 act through different pathogenetic pathways.<sup>24</sup> Moreover, within ABC DLBCL, patients with N1 mutation had worse progression-free survival and overall survival compared to patients with N2 mutation.<sup>24</sup> These data highlight that N1 and N2 mutations are genetically, phenotypically, and clinically different, suggesting the need to extend targeting Notch1 in these aggressive forms of B-cell malignancies.

Here we review some of the latest strategies to target Notch in hematological malignancies with emphasizing innovative approaches or experiences that translated pre-clinical observations into clinical trials (Figure 2).

**Targeting Extracellular NOTCH1.** Unlike Notch pathway activation in mutated T-ALL, CLL, MCL, the canonical activation of Notch signaling is mediated by ligand-mediated mechanisms.<sup>25,26</sup> Thus, given the role of Notch in several humans’ cancers, the development of therapeutic agents that interfere with ligand-receptor binding has seen a great impetus in the last years.<sup>27</sup>

A strategy that has been extensively explored is the development of antibodies (Abs) to block Notch ligand-receptor interaction. Several groups developed receptors-directed antibodies designed to antagonize NOTCH1, 2 and 3 by recognizing the NRR region of NOTCH to prevent the ADAM mediated metalloprotease cleavage.<sup>28-30</sup>

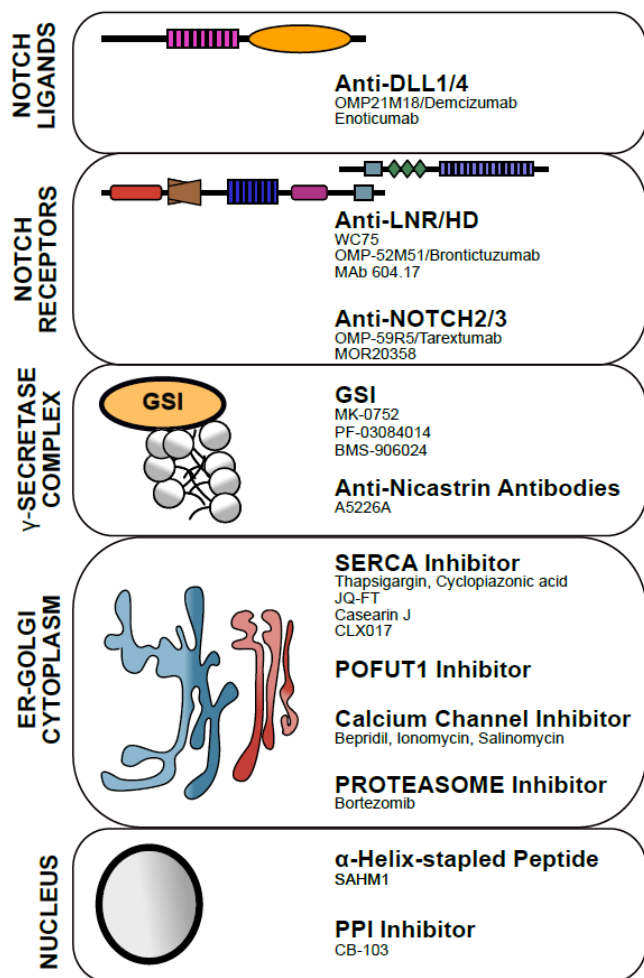
For example, Aste-Amezaga reported the

identification of two classes of NOTCH1 inhibitory monoclonal (m)Ab derived from cell-based and solid phase screening of a phage display library.<sup>31</sup> The first class comprises Abs directed to the EGF-repeat region (WC613), and the second directed to the NRR NOTCH1 domain (WC75). Both classes of antibodies inhibited canonical Notch signaling *in vitro* by repressing Notch transcriptional targets such as *Hes1* and *DTX1* genes. As predicted by the analysis of the putative NOTCH1 binding site, WC75 also inhibited Notch activation in a ligand-independent fashion such as in cancers mutated models (T-ALL), and similar to a  $\gamma$ -secretase inhibitor, Compound E, induced a gene expression signature consistent with Notch1 abrogation. Consistently WC75 inhibited the proliferation of NOTCH1 mutated T-ALL cell lines such as DND41 and KOPT-K1.<sup>31</sup>

Similarly OMP-52M51, a mAb generated by immunizing mice with a fragment of human NOTCH1 protein comprising the LNR plus the HD domain, efficiently blocked canonical Notch signal and reduced Notch activation in a series of T-ALL bearing HD and PEST mutations *in vitro* and in two patient-derived xenograft leukemia models carrying a L1679P mutation and a PEST deletion respectively.<sup>32</sup> In addition, OMP-52M51 prevented Notch1 activation in MCL cell lines *in vitro*.<sup>33</sup> OMP-52M51 (Brontictuzumab) was subsequently tested in a phase I dose escalation trial (NCT01778439) in patients with previously treated CLL, MCL, T-ALL, or other hematologic malignancies with known NOTCH1 mutational status. Of the 24 patients enrolled in this study, only five carried a NOTCH1 mutation, and just one of them achieved stable disease as the best response after 101 days of treatment. Overall OMP-52M51 was generally well tolerated but showed limited antitumor efficacy in this study.<sup>34</sup>

However, Sharma and colleagues further extended targeting NRR domain and reported the identification of the first mAb that recognizes clinically relevant mutant receptors.<sup>35</sup> The mAb 604.17 exhibited higher binding to mutant NOTCH1 compared to wild type and inhibited the proliferation of the T-ALL mutated cell line CCRF-CEM. Interestingly, 2  $\mu$ g/mL of mAb 604.17 preferentially inhibited the transcriptional activation of the NOTCH1 mutants L1549P, R1599P, and II1681N as assessed with a validated RPB1 12xCSL-luciferase promoter assay. Finally, 15 mg/kg of mAb 604.17 inhibited the tumor growth of different xenograft cancer models supporting the development of Notch mAbs as immunotherapeutic tools for different cancers.<sup>35</sup>

Besides Abs directed to NOTCH1 NRR domain, additional probes have been developed to NOTCH2 and NOTCH3.<sup>28</sup> For example, OMP-59R5 (Tarextumab), was generated by panning the HuCAL GOLD phage-display library with recombinant



**Figure 2.** The figure shows an overview of therapeutic targeting of Notch signaling.

NOTCH2 extracellular domain (EGF1–12) containing the ligand-binding site. OMP-59R5 showed antitumor activity in breast, ovarian, and small-cell lung cancer.<sup>36</sup> A phase Ib clinical trial showed that Tarextumab is well tolerated, and showed a dose-dependent biomarker-driven activity in patients with small-cell lung cancer (SCLC).<sup>37,38</sup>

The Blacklow laboratory leveraged the development of inhibitors and activators NRR mAbs to dissect the dynamics of NOTCH3 activation.<sup>39,40</sup> Given the prevalence of NOTCH3 activation (ICN3) and the recurrence of *NOTCH3* mutations in different cancer models, including T-ALL, the authors demonstrated that MOR20350 and MOR20358 inhibited Notch3 signaling *in vitro*.<sup>41,42</sup> MOR20350 and MOR20358 exhibited an anti-tumor effect using orthotopic xenograft models representative of cancer carrying a *NOTCH3* PEST (MDA-MB468) or NRR (TALL-1) mutations, respectively.<sup>41</sup>

A second strategy to inhibit Notch signaling is by developing Abs directed against Notch ligands such as DLL1 and DLL4.<sup>43</sup> For example, OMP-21M18 emerged from murine hybridoma library screen set to identify DLL4 inhibitors using a Notch-responsive luciferase reporter assay in HeLa cells.<sup>44</sup> DLL4 has a

unique role in regulating vascular endothelial cell proliferation and differentiation. Suppression of DLL4-mediated Notch signaling increases nonproductive angiogenesis but efficiently inhibited tumor growth in several cancer models.<sup>45</sup> However chronic inhibition of dll4 showed to alter normal liver endothelial histology in mice, rats, and cynomolgus monkeys and promotes subcutaneous vascular neoplasms in rats.<sup>46</sup> Despite safety concerns, OMP-21M18/Demcizumab entered clinical development, and it has been investigated in a phase I dose escalation and expansion study in patients with previously treated solid tumors (RGN-124, NCT01189929).<sup>47</sup> However, given the lack of clinical responses assessing the role of OMP-21M18 in combination with paclitaxel plus gemcitabine in treatment-naïve patients with metastatic pancreatic cancer OncoMed Pharmaceuticals discontinued ongoing demcizumab trials. Similarly to demcizumab, enoticumab a humanized IgG1 anti-Dll4 was tested in a phase I trial in ovarian cancers and solid tumors (48). Enoticumab was well tolerated (most of the patients experienced fatigue, headache, hypertension, and nausea) and response to treatment was confirmed in 2 out of 53 patients (5%) treated at 3 mg/kg (one patient with papillary serous ovarian carcinoma, and one patient with non-small cell lung cancer) while 16 patients (36%) had a stable disease.<sup>48</sup> Demcizumab, enoticumab trials are not extended to patients with hematological malignancies so far.

**Targeting the  $\gamma$ -Secretase Complex.** Because of its crucial role in Alzheimer's disease pathology,  $\gamma$ -secretase has been the target of many small molecules that were initially designed to reduce the generation of A $\beta$  polypeptides in the amyloid plaques. Among other substrates, the  $\gamma$ -secretase complex proteolyzes the release of ICN1 and therefore represents a critical step in the canonical Notch signaling. Thus, inhibitors of the  $\gamma$ -secretase complex (GSIs) that target all NOTCH receptors were re-purposed in cancers where *NOTCH1* mutations are common (T-ALL, CLL) and tumor dependency has been established in preclinical models. For example, in T-ALL, several studies showed that GSI treatment induces G0/G1 arrest along with rapid clearance of intracellular NOTCH1.<sup>49-52</sup>

De Angelo and collaborators completed the first GSI trial in T-ALL in six adults and two pediatric patients with leukemia (seven with T-ALL) treated in average for 56 days with MK-0752 a potent inhibitor developed by Merck & Co. In a T-ALL patient, with an activating *NOTCH1* mutation, the response was transient.<sup>53</sup> Overall, MK-0752 was poorly tolerated. In fact, most of the patients suffered from gastrointestinal toxicity, primarily diarrhea, observed at drug doses of 300 mg/m<sup>2</sup>. Subsequent studies showed that the gastrointestinal toxicity was due to the simultaneous blockade of NOTCH1 and NOTCH2 mediated by GSIs.

Abrogation of the Notch1/2 signaling in the gut leads to severe intestinal secretory metaplasia, an increase of goblet cells and a differentiation failure in the crypts of the small intestine<sup>54</sup> suggesting that targeted inhibition of individual receptors might reduce on-target gut toxicity.<sup>28</sup>

The MK-0752 failure, rushed for the identification of second generations GSIs with better tolerability profile and of combination strategies to overcome the limitation showed with the single drug treatment. Real and colleagues demonstrated that glucocorticoid therapy in combination with NOTCH1 inhibition by GSIs improved the antileukemic effect of GSIs and reduced their gut toxicity *in vivo*.<sup>55,56</sup> GSI sensitizes steroids resistant T-ALL cell lines and primary patients to glucocorticoid therapy and induced apoptosis through induction of *BCL2L11*. Mice treated with glucocorticoids and a GSI showed decreased gastrointestinal toxicity compared to animals treated with GSI alone. Steroids mediate the induction of cyclin D2 (CCND2), a cyclin associated with cell cycle progression, and by the down-regulation of Kruppel-Like Factor 4 (KLF4), a negative regulator of the cell cycle that is required for goblet cell differentiation.<sup>14,55</sup> In addition, Cullion and collaborators demonstrated that intermittent GSI dosing with drug holiday largely avoided gastrointestinal toxicity while maintaining efficacy in a mouse T-ALL model.<sup>57,58</sup>

However, gut toxicity is not the only off-target effect seen in GSI treated patients, raising additional concerns on chronic inhibition of wild-type NOTCH1. In two early-terminated phase III trials, LY450139 (semagacestat), failed to achieve the primary endpoints (improvement in the cognition and the ability to complete activities of daily living) in patients with mild-to-moderate Alzheimer's disease.<sup>59</sup> Data showed that semagacestat was associated with an increased risk of skin cancer compared with those who received placebo, likely due to inhibition of Notch in the skin by chronic GSI administration consistent with the tumor-suppressor role of Notch signaling in this tissue.<sup>60,61</sup> In addition, recent studies suggested that Notch signaling blockade might increase the risk of developing lung squamous cell carcinoma (SCC).<sup>62</sup> Whether this risk will be ameliorated by intermittent, pulsed therapy with GSI, as would be the schedule in cancer-directed therapy, is still to be determined.<sup>14</sup>

An additional GSI that reached clinical development is PF-03084014/Nirogacestat a noncompetitive, reversible GSI developed by Pfizer.<sup>63</sup> PF-03084014 induced an anti-leukemic effect *in vitro* and *in vivo* in T-ALL cell lines expressing mutant *NOTCH1*. An intermittent dosing schedule of PF-03084014 and the addition of glucocorticoids attenuated Notch-dependent gastrointestinal toxicity by reducing the loss of body weight in an HBP-ALL T-ALL xenograft model<sup>63</sup> confirming previous Cullion's

observations. PF-03084014 induces selective apoptosis in primary CLL cells carrying *NOTCH1* mutations and synergize with fludarabine in a stroma coculture model system.<sup>64</sup> In a phase I trial aimed to determine the safety profile and maximum tolerated dose (MTD) of PF-03084014, one out of eight relapsed/refractory T-ALL patients achieved a complete remission.<sup>65</sup>

Knoechel and colleagues reported a complete hematological response in a patient with early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) carrying a *NOTCH1* mutation treated with the GSI developed by the Bristol-Myers-Squibb 906024. A phase I trial, CA216002, confirmed this encouraging result and demonstrated the safety of BMS-906024 administered on weekly dosing (4-6 mg) in 25 pediatric patients with T-ALL or T-cell lymphoblastic lymphoma.<sup>66</sup> This study was the first trial reporting multiple responses to GSI inhibition, including a complete response and one partial response. Overall, 32% of the patients showed at least a 50% reduction in bone marrow (BM) blasts with tolerable side effects.<sup>66</sup> Interestingly, in pre-clinical studies, BMS-906024 enhanced the anti-leukemic activity of Ibrutinib in B-CLL cells *in vitro* by inhibiting ICN1 activation and consequently the transcription of its targets such as *c-MYC*.<sup>67</sup>

An alternative strategy to modulate  $\gamma$ -secretase activity is by developing mAbs directed to functional components of this complex. The  $\gamma$ -secretase complex comprises a catalytic core formed by presenilin 1 and presenilin 2 (PS1 and PS2) and three accessory proteins: anterior pharynx-defective 1 (APH-1), nicastrin (NCT), and presenilin enhancer protein 2 (PEN2).<sup>68</sup> For example, Hayashi and colleagues reported the identification of two mAbs A5226A and A5201A directed against the extracellular domain of NCT.<sup>69,70</sup> A5226A inhibited  $\gamma$ -secretase activity by competing with the NCT substrate binding *in vitro*. In addition, A5226A inhibited the proliferation of a *NOTCH1* mutated T-ALL cell line, DND41, and prevented ICN1 cleavage. In a xenograft model of DND41, A5226A administered at 50 mg/Kg/day reduced cancer cells growth *in vivo*.<sup>69</sup>

As discussed above, several GSIs showed preclinical activity and have entered late development,<sup>71</sup> limitations include lack of substrate selectivity and toxicities.<sup>72</sup> In addition, genetic and epigenetic mechanisms of resistance partially explained the lack of successful clinical translation on a large scale. To identify mechanisms of resistance to NOTCH1 inhibition in T-ALL, the laboratory of Dr. Ferrando analyzed the global gene expression signatures associated with a sensitivity of resistance to GSI. They demonstrated that the transcriptional suppression of *PTEN* was associated with resistance to GSI treatment in T-ALL cell lines. Protein analysis and mutation sequencing showed the absence, or the

marked reduction of PTEN at the protein level and biallelic *PTEN* mutation in resistant T-ALL cell lines.<sup>73,74</sup>

Knoechel and collaborators described an additional mechanism of tolerance to GSI therapy. In this work, the authors identified from *in vitro* long-term culture under GSI positive selections a subpopulation of GSI-tolerant T-ALL cells called “persister”. They described that resistance to GSI was reversible after the drug’s withdrawal; thus, they speculated the existence of an epigenetic mechanism of drug resistance. Therefore, they performed a short hairpin RNA (shRNA) screen targeting genes involved in chromatin regulation. Among top hits, which preferentially impaired the viability of “persister” cells while sparing the naïve population, they identified the BET (bromodomain and extra-terminal domain) family, *BRD4*. Consistently “persister” cells were more sensitive to BRD4 inhibition (JQ1) *in vitro* and combination therapy targeting “naïve” (GSI) and “persister” (JQ1) was significantly more effective in T-ALL xenotransplant models *in vivo*.<sup>75</sup>

**Targeting NOTCH Trafficking.** As we described above, NOTCH1 is a rational therapeutic target in several hematological malignancies, but as a mutated transcription factor, it poses a drug discovery challenge. Several groups contributed to the development of a program to overcome limitations associated with the targeting of transcription factors (e.g. *NOTCH1*)<sup>76-81</sup> or resistance to target therapy.<sup>82-84</sup> For example, we completed a gene expression-based high-throughput small molecule (GE-HTS)<sup>49,85</sup> and a cDNA overexpression screen using cell-based assays reporting Notch transcriptional activity.<sup>86</sup> To enrich for targets that preferentially impair NOTCH1 receptor bearing HD mutations (NRR), we deliberately selected to screen against a human T-ALL cell line (DND41), which carries a clinically relevant activating mutation in the HD of *NOTCH1* along with a PEST domain deletion (L1594PΔPEST) and secondly to identify gene products that would enhance the activation of a transcriptional reporter downstream of a mutant *NOTCH1* receptor frequently identified in T-ALL patients (L1601PΔPEST). Several ion flux modulators or genes encoding for ion channels or pumps scored as hits in the small molecules or the cDNA screens, respectively. One of the top compound hits was thapsigargin, an analog of thapsigargin, which is a non competitive inhibitor of the sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA). Among the top cDNA hits were *ATP2A1*, *ATP2A2*, and *ATP2A3*, which encode SERCA1, SERCA2, and SERCA3, respectively. We next showed that SERCA inhibition impairs the trafficking of mutated NOTCH1 receptors and induces a G0/G1 arrest in *NOTCH1*-mutated human T-ALL cells (**Figure 1B**). Thapsigargin had on-

target activity in mouse models of human T-ALL and also interfered with Notch signaling in *Drosophila*.<sup>76,87</sup> Remarkably, thapsigargin preferentially inhibited mutated NOTCH1 receptors.<sup>76</sup> This selectivity provides a therapeutic window not observed before with GSIs or most antibody-based approaches that are equipotent inhibitors of mutated and wild type (WT) receptors. Subsequent independent studies confirmed our original observation and demonstrated that thapsigargin alone or in combination with mAb 604.107 inhibited “gain of function” mutants associated with T-ALL such as L1594P, R1599P and I168N.<sup>35</sup>

Thapsigargin is an organic heterocyclic compound that is a hexa-oxygenated 6,7-guaianolide isolated from the roots of *Thapsia garganica*. Thapsigargin inhibits SERCA-mediated calcium ( $Ca^{2+}$ ) uptake leading to a depletion of the endoplasmic reticulum (ER)  $Ca^{2+}$  storage and sustained elevation of cytosolic  $Ca^{2+}$  triggering ER stress,<sup>76</sup> unfolded protein response (UPR), and different cellular pathways that can cause cell death. This general mechanism of cytotoxicity to develop SERCA inhibitors for cancer therapies has been leveraged. For example, SERCA has been identified as an emerging target in the treatment of prostate cancer.<sup>88</sup> SERCA channels are critical to maintaining intracellular  $Ca^{2+}$  homeostasis in all cell types. Thus, the direct delivery of thapsigargin to animals or humans might be expected to incur cardiac toxicity secondary to  $Ca^{2+}$  ion shifts. A strategy to prevent a systemic cytotoxic effect by inhibiting SERCA is by creating inactive pro-drugs that are activated in a histo-specific manner.<sup>89</sup> This, for example, is the mode of action of mipsagargin,<sup>90,91</sup> a TG soluble prodrug undergoing clinical trials for solid tumor.<sup>89</sup>

In the past, we imagined a general strategy for efficient TG delivery leveraging the dependency to folate metabolism of leukemia cells and developed a folate-TG derivative compound to transfer the inhibitor specifically to the T-ALL cells.<sup>92</sup> We showed that the 8-*O*-debutanoylthapsigargin, a cytotoxic TG analog, retained the anti-leukemia specificity toward mutant *NOTCH1* in T-ALL cell lines. Thus, we linked the carboxylate of folic acid to the C8-alcohol of 8-*O*-debutanoylthapsigargin, to generate the folate-thapsigargin conjugate named JQ-FT. We demonstrated that JQ-FT inhibits NOTCH1 *in vitro* in multiple T-ALL models and *in vivo* on a syngeneic T-ALL mouse model carrying a *NOTCH1* L1601P ΔPEST a common mutation observed in the human disease.<sup>92</sup> In the Notch arena, JQ-FT is the first-in-class NOTCH1 inhibitor with dual selectivity: leukemia over normal and NOTCH1-mutant over wild type receptors.

In the recent past, several putative SERCA inhibitors have been described. However, only a few have been tested in Notch-dependent diseases. Ford and colleagues demonstrated that the natural tricyclic

clerodane diterpene casearin J (CJ),<sup>93</sup> can affect the Notch1 pathway in human T-ALL cells. CJ reduced cell surface expression of NOTCH1 receptors, prevented the formation of the cleaved ICN1 molecules, which resulted in the transcriptional inhibition of Notch targets such as *MYC*, *HES1*. The authors showed that CJ inhibits SERCA protein causing a rise of intracellular Ca<sup>2+</sup> and depletion of the ER Ca<sup>2+</sup> storage. This ion shift concentration increases reactive oxygen species (ROS) and ultimately leads to apoptosis in T-ALL cells.<sup>93</sup> However, while the authors claimed selectivity toward HD-mutations, they did not demonstrate the lack of CJ activity in a large panel of wild type T-ALL models. In addition, it is not clear whether CJ causes an accumulation of full-length NOTCH1, as for other SERCA inhibitors,<sup>76</sup> suggesting that different interactions in the SERCA binding site may be responsible for the effect on Ca<sup>2+</sup> and consequently on Notch activation.

Ethyl 2-Amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (CXL017) is a recently synthesized SERCA inhibitor tested in multiple leukemia cell lines that acquired multidrug resistance through different mechanisms, including T-ALL.<sup>94</sup> Additional studies demonstrated that CXL017 synergizes with other SERCA inhibitors including thapsigargin and cyclopiazonic acid indicating that CXL017 may bind SERCA at a unique allosteric site<sup>95</sup> pointing to the potential of developing new classes of SERCA modulators.

In our original GE-HTS screen, multiple compounds reported modulating Ca<sup>2+</sup> ion flux scored as dose-dependent Notch pathway inhibitors including ionomycin, salinomycin, and bepridil.<sup>76</sup> Thus we initially extended testing the FDA approved Ca<sup>2+</sup> antagonist bepridil in T-ALL.<sup>96</sup> *In vitro*, bepridil reduced ICN1 and consequently caused a phenotype consistent with Notch abrogation in this tumor. While we can reach this effect at the plasma level concentration achievable in human, we did not demonstrate an effect in T-ALL orthographs, and we halted further experiments.<sup>96</sup> However, because we showed a transcriptional overlap between the *NOTCH1* “Off” signatures in T-ALL and CLL, we re-purposed bepridil for B-cell malignancies.<sup>97</sup> In CLL bepridil exerted an anti-leukemia activity *in vivo* associated with NOTCH1 inhibition.<sup>97</sup> Similar to thapsigargin, histological analysis of the gut showed normal goblet cell number with preservation of the architecture and proliferation of the intestinal epithelium suggesting a lack of combined NOTCH1 and NOTCH2 inhibition in this tissue. This result suggests Ca<sup>2+</sup> mediated inhibition of Notch signaling may overcome the limitation associated with  $\gamma$ -secretase inhibition. An additional strategy to alter NOTCH trafficking is by modulating the protein O-fucosyltransferase-1. POFUT1 catalyzes the addition of O-linked fucose to

the EGF-repeat domains of the NOTCH receptor that is required for NOTCH activation.<sup>98</sup> McMillan and colleagues showed that CRISPR/Cas9 mediated POFUT1 knockout in U2OS cells suppresses Notch activation signaling associated with type I and II mutations.<sup>99</sup> Interestingly, NOTCH1 protein does not mature in the CRISPR-engineered U2OS cells lacking POFUT1, a phenotype that mimics closely TG inhibition.

**Targeting NOTCH Degradation.** NOTCH is a short-lived protein and undergoes degradation mainly through an E3-ligase (Fbw7) ubiquitin-mediated pathway controlled by the PEST domain. As we described above, disruption of the PEST domain leads to an increase in ICN half-life.<sup>25</sup> In recent work, Koyama and colleagues demonstrated that the proteasome inhibitor, bortezomib, repressed the transcription of *NOTCH1* and of its downstream targets including *HES1*, *GATA3*, *RUNX3* and *CYLD* in MOLT4, JURKAT and CEM T-ALL cell lines.<sup>100</sup>

Drug combination studies revealed that bortezomib showed synergistic or additive effects with key drugs to treat T-ALL such as dexamethasone, doxorubicin, and cyclophosphamide. The synergistic effect of bortezomib and dexamethasone was confirmed at NOTCH1 protein expression level and later *in vivo* using a murine MOLT-4 T-ALL cell xenograft model.<sup>100</sup> This study supported the rationale of an ongoing clinical trial assessing the role of bortezomib in combination with different chemotherapy regimen (NCT02112916) in younger patients with newly diagnosed T-ALL or stage II-IV T-cell lymphoblastic lymphoma.

In parallel, Bertaina and colleagues tested bortezomib in combination with chemotherapy in 30 and 7 children with B-cell precursor (BCP) and T-cell ALL, respectively.<sup>101</sup> Bortezomib (1.3 mg/m<sup>2</sup>/dose) was administered intravenously twice a week x 2 with a chemotherapy regimen containing dexamethasone, doxorubicin, vincristine, and pegylated asparaginase. Twenty-two of 30 BCP-ALL patients (73.3%) and 5/7 patients (71%) with T-cell ALL achieved CR/CRp. The 2-year overall survival (OS) was 31.3% while patients that achieved an MRD response had a 2-year OS of 68.4%.<sup>101</sup> These data suggest that bortezomib may represent a clinically effective option in *NOTCH1* mutated T-ALL patients.

In CLL, Notch2 signaling appears to have a constitutive role in promoting cell survival and CD23 expression.<sup>102,103</sup> Several studies showed that B-CLL undergoes apoptosis upon proteasome inhibitors treatment.<sup>104,105</sup> However, Duecheler and colleagues demonstrated that bortezomib and MG132 efficiently induced apoptosis in B-CLLs *in vitro* by inhibiting NOTCH2 transactivation and repressing CD23 expression.<sup>106</sup> Similarly, in MCL, several studies

demonstrated the effects of proteasome inhibition on several intracellular mechanisms.<sup>107</sup> For example, bortezomib showed to induce cell cycle arrest and apoptosis by inhibition of NF- $\kappa$ B,<sup>108</sup> inhibition of the protein kinase CK2,<sup>109,110</sup> the depolarization of the mitochondria membrane, ROS release, and the production of pro-apoptotic proteins (NOXA).<sup>111</sup> In addition, several pre-clinical studies demonstrated the synergist activity of bortezomib with other antineoplastic agents<sup>112,113</sup> including the HDAC inhibitor vorinostat (SAHA),<sup>114</sup> idelalisib,<sup>115</sup> and the anti-CD20 mAb rituximab.<sup>116</sup> While many clinical trials confirmed that combining bortezomib with other anti-lymphoma therapies is feasible effective none at the moment focused on the role of Notch signaling mediating the efficacy or resistant to therapy.

**Targeting ICN1 Complex.** As described above, activation of NOTCH1 receptor results in a sequence of cleavages that cause the release of ICN1. Following translocation to the nucleus, ICN1 forms a ternary complex with the transcriptional repressor CSL (CBF-1, Suppressor of Hairless and Lag-1) co-activators of the Mastermind-like family (MAML1-3 in humans) bound to DNA. Thus, Moellering and colleagues developed a cellular penetrant, soluble  $\alpha$ -Helix-constrained “stapled” peptide derived from mastermind-like 1, SAHM1 that can bind the ICN-CSL complex. Similarly to GSI, SAHM1 produced a transcriptional signature of *NOTCH* gene repression in human and murine T-ALL cells. Direct blockade of NOTCH-CSL transcriptional complex reduced NOTCH-specific anti- proliferative effects in human T-ALL cell lines and in a bioluminescent murine model of T-ALL.<sup>117</sup>

While this approach holds the premises to be more specific for Notch compared to GSIs, which also affect the cleavage of different cellular substrates, its clinical translation is hampered by the lack of pharmacokinetics and pharmacodynamics studies.

Recently Cellestia Biotech AG developed CB-103 a small molecule protein-protein interaction (PPI) inhibitor able to target assembly of the Notch transcription complex in the cell nucleus leading to down-regulation of Notch target genes (*c-MYC*, *CCND1*, *HES1*) and inhibition of Notch signaling independently of Notch mechanisms of activation. This pan-Notch inhibitor has shown preclinical activity in a variety of solid tumors and leukemia models. In preclinical studies CB-103 inhibited the proliferation of

various cancer cell lines including T-ALL with known *NOTCH1* mutational status (RPMI-8402 and KOPT-K1) compared to the GSI 4929097. Both ICN1, transmembrane NOTCH1 and full-length decrease upon CB-103 treatment consistent with a mechanism of transcriptional inhibition.<sup>118</sup> Spriano and colleagues extended testing CB-103 in a collection of 61 B and T cell lymphoma cell lines. CB-103 presented a median IC50 above 20  $\mu$ M across the whole panel of lymphoma cell lines (range from 400 nM to > 20  $\mu$ M), without significant differences among lymphoma subtypes.<sup>119</sup> Sensitive lines (IC50 < 10  $\mu$ M) presented a gene expression signature significantly enriched with genes involved in the epithelial-mesenchymal transition, a Notch-related process.<sup>119</sup> A multicenter open-label, non randomised phase I-II clinical trial (CB-103-C-101) is ongoing enrolling patients with advanced, refractory or metastatic solid tumors and hematological malignancies for whom no standard therapy exists.<sup>120</sup> Notch mutational status or expression is not key inclusion criteria of the study but it stands among the exploratory analysis suggesting that, as in other previous studies, responses in Notch mutated cases may be few.

**Conclusions.** In the last two decades, we have seen significant improvements in T-ALL, CLL and MCL survival. However, a significant number of patients relapse or rapidly became resistant to available therapeutic options. Thus, the development of a Notch targeted approach appears a rational strategy to modulate a pathway on which these cancer cells rely on to survive. Despite  $\gamma$ -secretase inhibitors experienced several roadblocks in their development we are achieving a better characterization of disease's pathways that will facilitate the development of mutant selective of context-dependent inhibitors for these aggressive tumors. Furthermore, the development of Notch isoform selective small molecules along with re-defined therapeutic schedule will overcome the hurdle associated with the off-target toxicities seen with the chronic inhibition of wild type NOTCH1 and NOTCH2.

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