A novel small molecule inhibitor of the Notch transcription activation complex.

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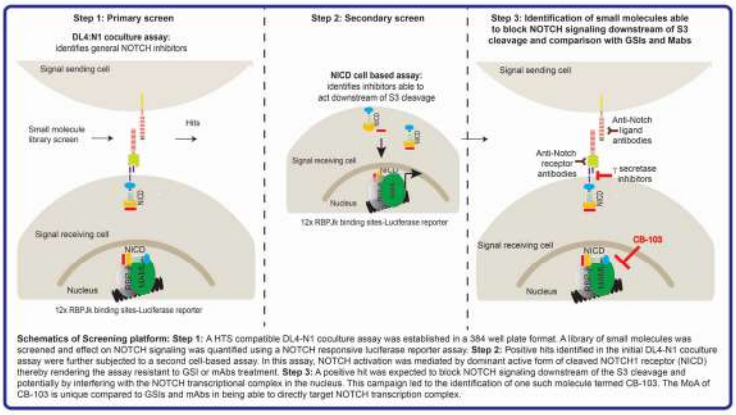
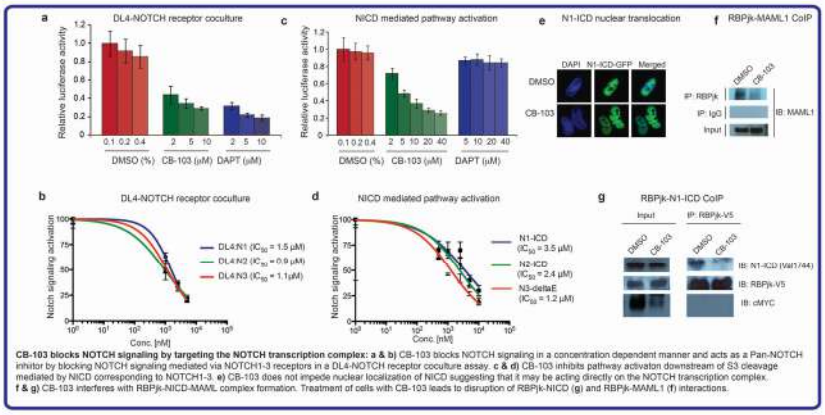
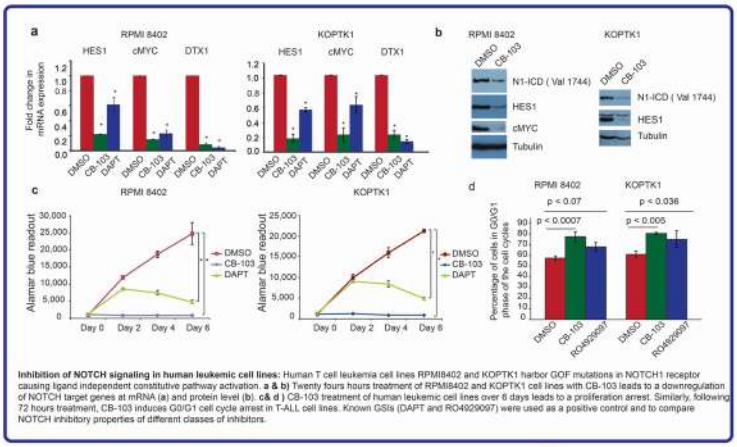
Abstract: NOTCH signaling is a developmental pathway known to play critical roles in the regulation of self-renewing tissues. Aberrant activation of NOTCH signaling leads to deregulation of the self-renewal process resulting in sustained proliferation, evasion of cell death, loss of differentiation capacity, invasion and metastasis, all of which are hallmarks of cancer. Given the importance of NOTCH signaling in human cancers,several therapeutic approaches have been utilized to block NOTCH signaling and have confirmed it as a therapeutic target. Two of these strategies are; a) the use of monoclonal blocking antibodies (mAbs) against NOTCH ligands and receptors and b) the use of small molecule γ-secretase inhibitors (GSIs). A third, yet not fully explored approach could be the blockage of NOTCH signalling by targeting the most downstream event in the NOTCH cascade i,e NOTCH transcriptional activation complex using small molecule inhibitors.
Here we report discovery and identification of CB-103, a novel, orally-active small molecule inhibitor of the NOTCH pathway. CB-103 blocks NOTCH signaling by targeting the NOTCH transcriptional activation complex in the nucleus. CB-103 inhibits NOTCH signaling in human cancer cell lines with activated NOTCH pathway, induces neurogenic phenotype in drosophila, induces satellite cell differentiation and inhibits NOTCH mediated differentiation processes in mice (e.g Marginal Zone B cells). In addition, CB-103 exhibit anti-tumor efficacy in various *in vivo* models , including xenograft model of human triple negative breast cancer resistant to GSIs and mAb against NOTCH ligands/receptors. Furthermore, CB-103 has shown a remarkable activity in PDX models of human T-ALL harboring activation of the NOTCH pathway and on *ex vivo* treated patient-derived leukemic samples.



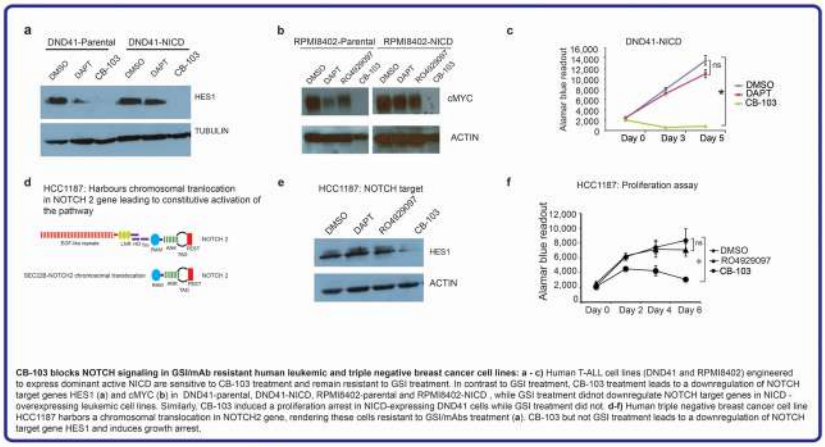
Schematics of Screening platform: Step 1: A HTS compatible DL4-N1 coculture assay was established in a 384 well plate format. A library of small molecules was screened and effect on NOTCH signaling was quantified using a NOTCH responsive luciferase reporter assay. **Step 2:** Positive hits identified in the initial DL4-N1 coculture assay were further subjected to a second cell-based assay. In this assay, NOTCH activation was mediated by dominant active form of cleaved NOTCH1 receptor (NICD) thereby rendering the assay resistant to GSI or mAbs treatment. **Step 3:** A positive hit was expected to block NOTCH signaling downstream of the S3 cleavage and potentially by interfering with the NOTCH transcriptional complex in the nucleus. This campaign led to the identification of one such molecule termed CB-103. The MoA of CB-103 is unique compared to GSIs and mAbs in being able to directly target NOTCH transcription complex.



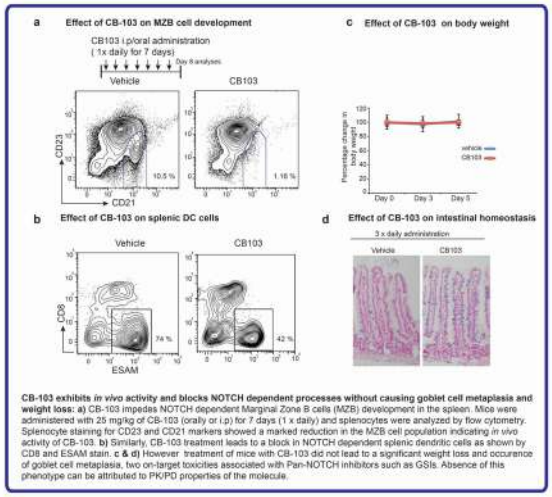
CB-103 blocks NOTCH signaling by targeting the NOTCH transcription complex: a & b) CB-103 blocks NOTCH signaling in a concentration dependent manner and acts as a Pan-NOTCH inhibitor by blocking NOTCH signaling mediated via NOTCH1-3 receptors in a DL4-NOTCH receptor coculture assay. **c & d)** CB-103 inhibits pathway activaton downstream of S3 cleavage mediated by NICD corresponding to NOTCH1-3. **e)** CB-103 does not impede nuclear localization of NICD suggesting that it may be acting directly on the NOTCH transcription complex. **f & g)** CB-103 interferes with RBPjk-NICD-MAML complex formation. Treatment of cells with CB-103 leads to disruption of RBPjk-NICD (**g**) and RBPjk-MAML1 (**f**) interactions.



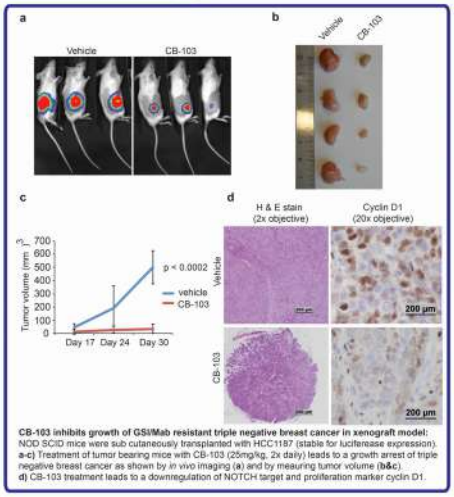
Inhibition of NOTCH signaling in human leukemic cell lines: Human T cell leukemia cell lines RPMI8402 and KOPTK1 harbor GOF mutations in NOTCH1 receptor causing ligand independent constitutive pathway activation. **a & b)** Twenty fours hours treatment of RPMI8402 and KOPTK1 cell lines with CB-103 leads to a downregulation of NOTCH target genes at mRNA (**a**) and protein level (**b**). **c& d)** CB-103 treatment of human leukemic cell lines over 6 days leads to a proliferation arrest. Similarly, following 72 hours treatment, CB-103 induces G0/G1 cell cycle arrest in T-ALL cell lines. Known GSIs (DAPT and RO4929097) were used as a positive control and to compare NOTCH inhibitory properties of different classes of inhibitors.



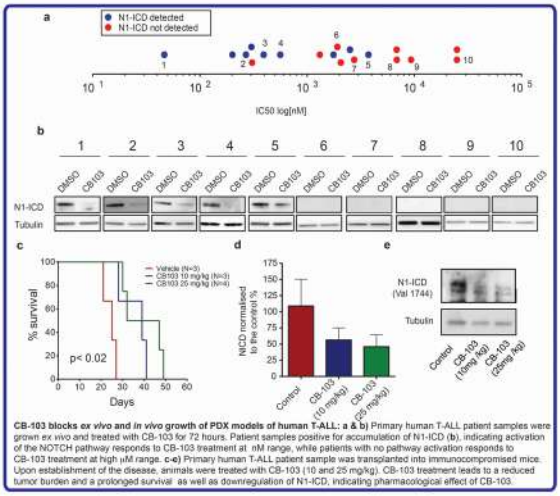
CB-103 blocks NOTCH signaling in GSI/mAb resistant human leukemic and triple negative breast cancer cell lines: a - c) Human T-ALL cell lines (DND41 and RPMI8402) engineered to express dominant active NICD are sensitive to CB-103 treatment and remain resistant to GSI treatment. In contrast to GSI treatment, CB-103 treatment leads to a downregulation of NOTCH target genes HES1 (**a**) and cMYC (**b**) in DND41-parental, DND41-NICD, RPMI8402-parental and RPMI8402-NICD , while GSI treatment didnot downregulate NOTCH target genes in NICD - overexpressing leukemic cell lines. Similarly, CB-103 induced a proliferation arrest in NICD-expressing DND41 cells while GSI treatment did not. **d-f)** Human triple negative breast cancer cell line HCC1187 harbors a chromosomal translocation in NOTCH2 gene, rendering these cells resistant to GSI/mAbs treatment (**a**). CB-103 but not GSI treatment leads to a downregulation of NOTCH target gene HES1 and induces growth arrest.



CB-103 exhibits *in vivo* activity and blocks NOTCH dependent processes without causing goblet cell metaplasia and weight loss: a) CB-103 impedes NOTCH dependent Marginal Zone B cells (MZB) development in the spleen. Mice were administered with 25 mg/kg of CB-103 (orally or i.p) for 7 days (1 x daily) and splenocytes were analyzed by flow cytometry. Splenocyte staining for CD23 and CD21 markers showed a marked reduction in the MZB cell population indicating *in vivo* activity of CB-103. **b)** Similarly, CB-103 treatment leads to a block in NOTCH dependent splenic dendritic cells as shown by CD8 and ESAM stain. **c & d)** However treatment of mice with CB-103 did not lead to a significant weight loss and occurence of goblet cell metaplasia, two on-target toxicities associated with Pan-NOTCH inhibitors such as GSIs. Absence of this phenotype can be attributed to PK/PD properties of the molecule.



CB-103 inhibits growth of GSI/Mab resistant triple negative breast cancer in xenograft model: NOD SCID mice were sub cutaneously transplanted with HCC1187 (stable for luciferase expression). **a-c)** Treatment of tumor bearing mice with CB-103 (25mg/kg, 2x daily) leads to a growth arrest of triple negative breast cancer as shown by *in vivo* imaging (**a**) and by measuring tumor volume (**b&c**). **d)** CB-103 treatment leads to a downregulation of NOTCH target and proliferation marker cyclin D1.



CB-103 blocks *ex vivo* and *in vivo* growth of PDX models of human T-ALL: a & b) Primary human T-ALL patient samples were grown *ex vivo* and treated with CB-103 for 72 hours. Patient samples positive for accumulation of N1-ICD (**b**), indicating activation of the NOTCH pathway responds to CB-103 treatment at nM range, while patients with no pathway activation responds to CB-103 treatment at high μM range. **c-e)** Primary human T-ALL patient sample was transplanted into immunocompromised mice. Upon establishment of the disease, animals were treated with CB-103 (10 and 25 mg/kg). CB-103 treatment leads to a reduced tumor burden and a prolonged survival as well as downregulation of N1-ICD, indicating pharmacological effect of CB-103.

Conclusions: Cellestia Biotech and EPFL's drug discovery program has led to the discovery and development of a novel chemical series of pharmacological inhibitors of the NOTCH pathway for which the current Development Candidate is CB-103. Our studies demonstrate that CB-103 inhibits NOTCH signaling through a unique mechanism of action. CB-103 blocks NOTCH signaling downstream of S3 cleavage of NOTCH receptors by directly targeting NOTCH transcription complex in the nucleus. Due to its novel mechanism of action, CB-103 effectively blocks NOTCH signaling mediated by dominant active forms of NICD, thus enabling application of CB-103 in human tumors driven by GOF mutations in the NOTCH receptors and by chromosomal translocation in NOTCH receptor genes (~9% TNBC) This will allow an application of CB-103 in GSI and mAbs (targeting NOTCH ligands and receptors) resistant human tumors. In addition, CB-103 does not exhibit goblet cell metaplasia and body weight loss at therapeutic active doses, two known on-target toxicities associated with Pan-NOTCH inhibitors such as GSIs. This advantage is related to the PK/ADME profile of CB-103, which shows that the compound is orally available and has a short in vivo half-life, allowing for highly flexible dosing regimens. CB-103 is currently undergoing IND-enabling toxicology studies, and FIM studies have been planned for Q1 2017. CB-103 has also been tested in combination with SOC chemotherapy regimen and targeted therapies.

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