

## Review

# Notch Signaling Pathway as a Therapeutic Target in Breast Cancer

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## Abstract

The highly conserved Notch signaling pathway is involved in regulating a number of key cellular processes. This pathway has been implicated in both the development and progression of breast cancer and has emerged as a possible therapeutic target. Several clinical trials are currently underway to determine if targeting the Notch pathway with drugs such as the  $\gamma$ -secretase inhibitors may be an effective therapeutic strategy that improves outcomes in this disease. *Mol Cancer Ther*; 10(1); 9–15. ©2010 AACR.

## Introduction

Breast cancer is the most common female cancer in the United States, the second leading cause of cancer death after lung cancer, and the main cause of death in women aged 20 to 59. In 2010, approximately 207,000 American women will be diagnosed with breast cancer and despite early detection and improved treatments almost 40,000 will die of it (1). The Notch signaling pathway has been implicated in the pathogenesis of breast cancer and as such may represent a novel therapeutic target.

## Overview of Notch Signaling

### Notch ligands

There are 5 Notch ligands, Delta-like (Dll) 1, 3, 4, and Jagged (JAG)1, 2, which are single transmembrane proteins, containing a characteristic extracellular DSL domain that mediates receptor binding, and multiple EGF-like repeats. Jagged ligands have an extra cysteine-rich domain, which is not present in the Delta-like ligands. The cytoplasmic regions of these ligands are not well characterized except for the C-terminal domain that contains a PDZ-binding motif (see refs. 2–5 for reviews).

### Notch receptors

There are 4 Notch transmembrane receptors: Notch 1–4, which are synthesized individually from independent

mRNAs as single protein precursors that undergo glycosylation by the enzyme protein O-fucosyl transferase in the endoplasmic reticulum. Some of the O-fucose moieties are further elongated by Fringe glycosyltransferases (Lunatic, Manic, and Radical), which modify the specificity of the receptor for its ligand (6). Notch receptors are then cleaved by the protease furin in the trans-Golgi network into 2 noncovalently linked domains, the notch extracellular domain and the notch intracellular domain (NICD). The notch extracellular domain contains a variable number of epidermal growth factor (EGF)-like repeats (between 26 and 29 depending on the Notch receptor); 3 LNR (LIN12/Notch related) repeats, which prevent ligand-independent signaling), and 2 conserved cysteine residues. The C-terminal transactivation domain contains a PEST sequence that facilitates rapid proteolytic degradation of the protein.

### Notch signaling

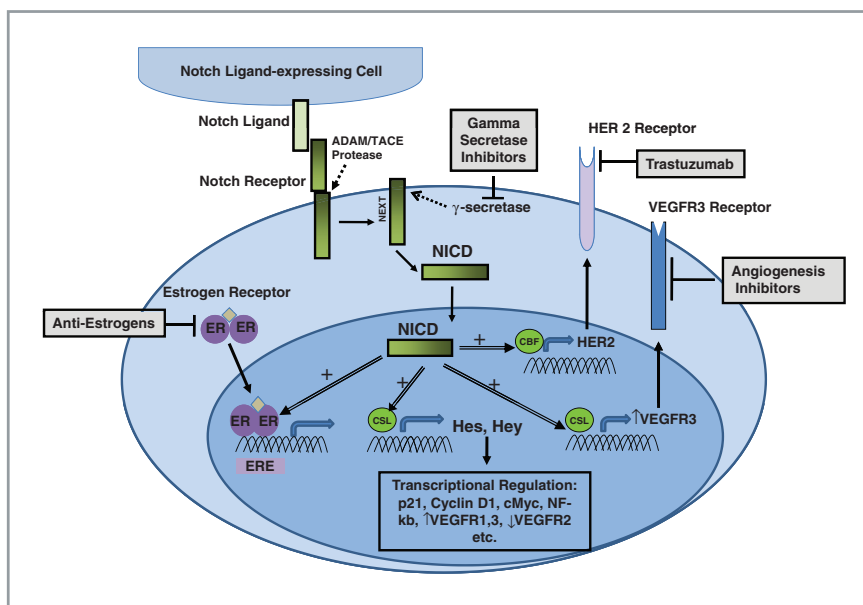
All 4 Notch receptors use the same basic signaling pathway that is activated by binding of Notch ligand on one cell to the extracellular domain of a Notch receptor on a neighboring cell. The Notch ligand–receptor complex then undergoes several key proteolytic cleavages. The cleavage is initially mediated by the ADAM/TACE family of proteases and occurs at an extracellular site (S2), between Ala (1710) and Val (1711) residues, approximately 12 amino acids outside the transmembrane domain. This generates a product known as NEXT (notch extracellular truncation), which is then cleaved by the  $\gamma$ -secretase complex, which consists of 2 key proteins, presenilin and nicastrin. Presenilin is the catalytic component of  $\gamma$ -secretase, whereas nicastrin is not catalytically active but promotes the maturation and proper trafficking of other proteins in the complex (7).  $\gamma$ -Secretase cleaves NEXT, which is the critical step that releases NICD that translocates into the nucleus and associates with CSL [CBF-1 (C-promoter binding factor 1), Suppressor of Hairless and Lag-1], a constitutive transcriptional repressor (8–11). After Notch binding, CSL becomes a

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**Figure 1.** The membrane-tethered Notch receptor is activated by binding to a ligand on a neighboring cell. This binding results in an initial cleavage triggered by the ADAM17/TACE metalloprotease, resulting in the generation of the NEXT product, which is further cleaved by  $\gamma$ -secretase, resulting in the release of the intracellular domain of Notch (NICD). NICD translocates to the nucleus, causing transactivation of downstream target genes including several genes (Hes, Hey). A number of signaling pathways may interact with Notch in the transformation of breast epithelial cells. These include the ER pathway, signaling downstream of Her2 and the VEGFR. Pharmacologic inhibitors of these pathways in combination with GSI are being tested in the context of breast cancer (8–11).

transcriptional activator and, in conjunction with co-factors such as mastermind-like (MAML) proteins, induces transcription of downstream targets including several Hairy/Enhancer of Split related genes (Hes, Hey), pT $\alpha$ , and Notch1 itself (12, 13). Both Hes and Hey proteins contain a basic domain, which determines DNA binding specificity and a helix-loop-helix domain, which allows for the formation of homo- or heterodimers. Either by interacting with co-repressors or by sequestering transcriptional activators, dimers of hes and/or hey proteins regulate the transcription of key genes (2, 14). These transcriptional targets include cell-cycle regulators (p21 and cyclin D1), transcription factors (c-Myc, NF-Kb2), and growth factor receptors (HER2) and regulators of angiogenesis and apoptosis (refs. 15–21; Fig. 1). Disruption of the Notch pathway can therefore have significant downstream effects on cell growth, differentiation, angiogenesis, and apoptosis.

### Notch Signaling and Tumorigenesis

The first indication that Notch plays a role in tumorigenesis came from the identification of the t(7:9)(q34; q34.3) chromosomal translocation in a subset of human pre-T-cell acute lymphoblastic leukemias (T-ALL). This translocation resulted in a truncated and constitutively active Notch 1 receptor under the control of the T-cell receptor beta promoter (TCR-B). Subsequently, activating mutations in Notch 1 have been discovered in more than 50% of human T-ALL cases (22, 23). Abnormalities in

various components of the Notch pathway have also been found in solid tumors (24–27).

In murine mammary cancers, the Notch 4 locus is a common proviral integration site for the MMTV (mouse mammary tumor virus), which induces mammary adenocarcinomas (28). MMTV insertion results in constitutive, ligand-independent expression of Notch 4 ICD and increased activation of Notch target genes. Human breast cancer cell lines have also been tested for Notch expression: A truncated and activated form of Notch 4 has been found in 2 of 8 cell lines and an activated Notch 1 ICD in 8 of 8 cell lines tested (29, 30). Notch 3 seems to play a role specifically in the proliferation of Erb2-negative breast cancer cell lines (31).

Studies in primary human breast cancers have shown that high-level expression of Jag1 (Jag1<sup>High</sup>) and/or Notch1 (Notch1<sup>High</sup>) mRNA in tumors correlates with poor outcome and is an independent prognostic indicator (32–34). It has also been shown that NUMB, a key negative regulator of the Notch pathway, is lost in greater than 50% of tumors due to ubiquitination and proteosomal degradation, and this also correlated with higher grade tumors (35).

### Notch Signaling and Cross Talk

The oncogenic role of Notch in breast cancer may be mediated in part through its cross talk with other signaling pathways, such as the estrogen pathway. Approximately 80% of breast cancers express the estrogen

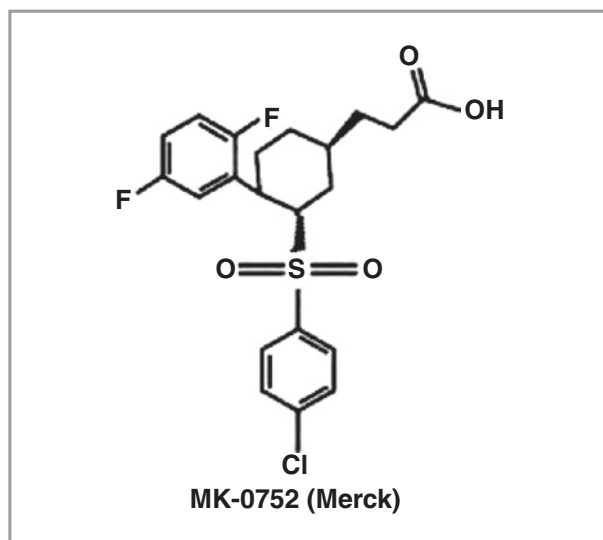


Figure 2. Chemical structure of the GSI MK-0752 (Merck; ref. 81).

receptor and are treated with anti-estrogens, but resistance to anti-estrogens often develops. One mechanism of resistance may be via the Notch pathway. In the absence of estrogens, Notch signaling becomes activated and can directly stimulate estrogen receptor  $\alpha$ -dependent transcription, overriding the inhibitory effects of anti-estrogens (36). From a therapeutic standpoint, concurrently targeting both the estrogen receptor and the notch pathway may help to overcome or at least in part delay this resistance.

The Notch pathway may also interact with the human epidermal growth factor receptor 2 (HER2) signaling pathway, which is active in approximately 20% of breast cancers and associated with a more aggressive disease. CBF-1 (which forms a complex with NICD) has been shown to have binding sequences in the HER2 promoter and at the same time inhibition of Notch signaling seems to downregulate Her2 expression. Taken together, this suggests an important link between these 2 pathways (37). Using HER2-targeted agents, such as trastuzumab or lapatinib in combination or in sequence with Notch pathway inhibitors, may therefore be a strategy that warrants further study.

Notch is also involved in angiogenesis, which is critical for tumor growth and proliferation. Zeng et al. have shown that Notch signaling from tumor cells can trigger Notch activation of neighboring endothelial cells and promote tumor angiogenesis (38). Notch signaling itself seems to increase levels of vascular endothelial growth factor receptors (VEGFR1,3; VEGFR3 the upstream promoter of VEGFR3 contains Notch-responsive CSL elements) and decreases VEGFR2 expression (39–41). Conversely, VEGF may also directly regulate expression of the Notch ligand Dll4 in tumor vessels. It has been shown that Dll4 levels correlate with VEGF levels and VEGF blockade results in a rapid and profound reduction

of Dll4 expression (42, 43). In a small study of 19 patients with metastatic breast cancer treated with 1 dose of the antiangiogenic agent bevacizumab, biopsies taken before and after treatment show increased expression of both VEGF and Notch target genes (hes and hey), again supporting an interaction between these 2 pathways (44). Concurrently, targeting both the notch pathway and the angiogenic pathway could therefore be explored further as long as toxicity is not a major problem. Notch may also cooperate with the ERK pathway (45). Constitutively active Notch 1 requires the ERK pathway to mediate transformation of immortalized breast cells, and activated Notch-positive tumors expressing phospho-Erk1/2 in the nuclei showed high node positivity. This suggests that Notch-Erk cooperation may not only be necessary for disease progression but also may lead to more aggressive disease (46). Tumors overexpressing H-Ras (either due to H-ras mutations or upstream growth factor receptor signaling) showed increased expression of Notch 1, indicating that Notch may also be a downstream effector of oncogenic Ras. Inhibiting Notch signaling seemed to suppress Ras-induced tumorigenesis, supporting a link between these pathways and a rationale for targeting both (47, 48).

Interactions between Notch and the Akt, TGFB, Wnt, and HIF pathways may also exist and as novel agents targeting these pathways become available, combination approaches with Notch inhibitors could be considered (47, 49, 50).

### Notch and Tumor-Initiating Cells

In breast cancers and other cancers, there is now increasing support for the theory that a subpopulation of cancer cells exist known as tumor-initiating cells (TIC) or cancer stem cells. These cells are not only capable of self-renewal and proliferation but have also been implicated in both treatment resistance and disease relapse (51–53). A population of CD24<sup>-</sup>/low/CD44<sup>+</sup> cells, believed to represent TICs, has been isolated from breast cancers and are 1,000 times more tumorigenic than cell populations lacking these cells, with injection of as few as 200 TICs causing tumor formation in SCID mice (51). TICs, like normal stem cells, are dependent on a number of key signaling pathways including the Notch pathway. Using mammospheres (in which putative mammary stem cells are cultured *in vitro* within multicellular spheroids), Dontu et al. have shown that the self-renewal capacity of mammospheres is enhanced 10-fold when cultured in the presence of a synthetic peptide derived from the DSL (delta-Serrate-Lag2) domain, which is highly conserved in all Notch ligands and capable of Notch receptor activation. Conversely, mammosphere self-renewal was inhibited by Notch 4 blocking antibody or an inhibitor of the  $\gamma$ -secretase enzyme (54). Similar findings have been reported by Farnie et al., who have shown that the efficiency of ductal carcinoma *in situ* derived mammosphere production was significantly

reduced when Notch signaling was inhibited (55). In primary breast cancer and breast cancer cell line–derived tumorspheres, Notch 3 and Jag1 have emerged as key regulators of TIC renewal and hypoxia survival (56, 57). Taken together, it would seem that targeting the Notch pathway might be one strategy to specifically target TICs, which may be more resistant to conventional anticancer treatments.

### Notch and Triple-Negative Breast Cancer

Triple-negative (TN) breast cancers represent about 20% of all breast cancers, and despite initial response to systemic treatment, this disease follows an aggressive course. Cell line data show that basal/TN cancers have elevated Jag 1 levels, and BRCA-1 mutant breast cancers, which are typically of the basal/TN subtype, show elevated Jag1 expression compared with their BRCA2 (predominantly luminal) counterparts (58). Resection specimens from TN breast cancers show a statistically significant association between elevated expression of Notch ligands/receptors and the basal/TN subtype (32, 34, 59). In a disease with a paucity of treatments, targeting the Notch pathway is currently under investigation.

### Therapeutic Implications of Notch Inhibitors

There is now significant interest in developing therapies targeting the Notch pathway in breast cancer. A number of genetic and pharmacologic approaches are either available or theoretically possible to block Notch signaling at different levels of the cascade. Notch receptors and ligands may be inhibited by selective strategies including monoclonal antibodies, antisense, or RNA interference; nonselective strategies including soluble ligands, receptor decoys; or inhibition of enzymes involved in glycosylation or cleavage of receptors, such as  $\gamma$ -secretase inhibitors (GSIs) or ADAM inhibitors, are also being explored.

At the present time, the GSIs originally developed as potential inhibitors of the presenilin  $\gamma$ -secretase complex that cleaves B-amyloid peptide (which leads to Alzheimer's disease through plaque formation) are the furthest in development as potential anticancer agents (60, 61). GSIs show antitumor activity in several human cancer cell lines. Xenograft studies with glioblastoma and lung adenocarcinoma cell lines have shown that GSIs reduced both tumor growth and vasculature, induced growth arrest of T-ALL cells, and induced apoptosis in melanoma cell lines (62–66). They have also been shown to effectively induce apoptosis in TN MDA-MB-231 cells. In ER<sup>+</sup> MCF7 cells, enhanced killing was seen when GSIs were combined with the anti-estrogen tamoxifen, suggesting that antiestrogen treatment in ER<sup>+</sup> cells may activate Notch signaling, which is then blocked by concurrent treatment with a GSI inhibitor (67). GSI treatment of numb-deficient

*in vitro*–cultured tumor explants resulted in both, decreased cell proliferation (as measured by Ki67) and decreased expression of the glucose transporter Glut1, suggesting positron emission tomographic (PET) imaging could be one modality used to measure response to GSI treatment (68, 69). Preclinical studies in MDA-MD-231 breast cancer cells have also shown that GSI when combined with ionizing radiation may have additive effects (70). Like other small-molecule inhibitors, GSIs have multiple downstream effects by targeting all Notch receptors, some ligands, ErbB4, syndecan, CD44, and other proteins. As a result, determining Notch pathway activity alone may not be the best predictor of response and it will be critical to develop biomarkers that accurately predict sensitivity to the GSIs. Mechanism-based toxicities will also have to be addressed by a careful choice of therapeutic agents, combinations, and regimens (71, 72).

In a phase 1 trial of the GSI MK-0752 (Merck) shown in Figure 2, given to 7 patients with advanced solid tumors and 14 patients with advanced breast cancer, the main adverse effects of continuous dosing included diarrhea, constipation, nausea, and abdominal cramping. Intermittent dosing schedules are now being investigated. Importantly, MK-0752 at all doses inhibited  $\gamma$ -secretase with a decrease in plasma A $\beta$ 40 (product of  $\gamma$ -secretase cleavage) by 46% at 4 hours on day 1 compared with predose levels (73). There are several ongoing clinical studies involving MK-0752 in breast cancer including 1 study exploring different dosing schedules, a study of MK-0752 in combination with tamoxifen or letrozole to treat early-stage breast cancer, and a phase I/II study of MK-0752 followed by docetaxel in advanced or metastatic breast cancer (74–76).

Another GSI, RO4929097 (Roche), shown in Figure 3, has also entered clinical trials. This drug has been evaluated in 2 dosing regimens—days 1–3, 8–10 every 3 weeks, and days 1–7 every 3 weeks, and found to be generally well tolerated, with the most common adverse events being reversible fatigue, nausea, emesis, diarrhea, hypophosphatemia, and rash. In this study, pretreatment IL-6 and IL-8 levels have emerged as possible response predictive markers and will be

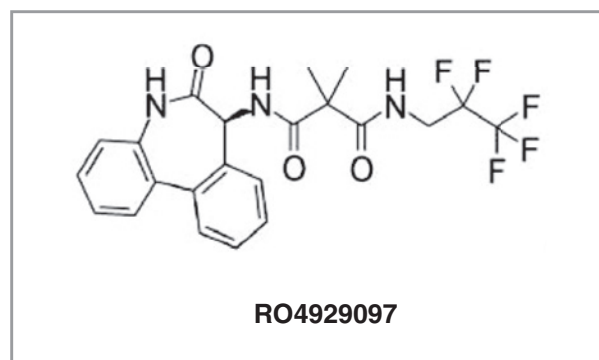


Figure 3. Chemical structure of the GSI RO4929097 (Roche; ref. 82).

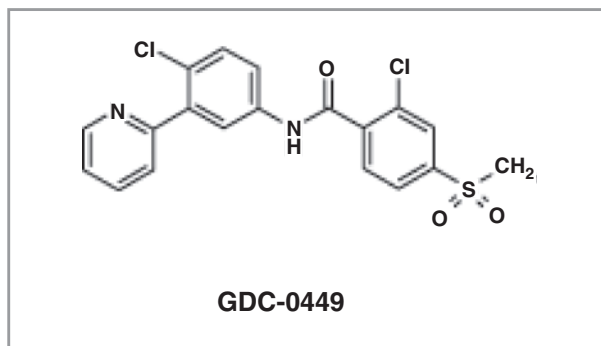


Figure 4. Chemical structure of the hedgehog inhibitor GDC-0449 (Genentech; ref. 83).

explored further (77). There is currently a phase II study in advanced TN breast cancer in which baseline and 4 to 5 week post-treatment tumor biopsies will be collected to explore the effect of the drug on components of the Notch pathway and surrogate markers of Notch inhibition. Drug exposure will also be explored through paired blood sampling. Another study combines R04929097 with the hedgehog inhibitor GDC-0449 (Genentech; Fig. 4) in advanced breast cancer (78). This combination is based on the theory that both the notch

pathway and the hedgehog signaling pathway play important roles in self-renewal pathways and also interact with one another (79). A potential challenge of targeting self-renewal pathways is clearly to ensure that normal cellular pathways dependent on self-renewal remain unaffected (80).

In conclusion, there is growing evidence that cross talk between Notch and key signaling pathways, as well as its role in regulating TICs, can promote tumorigenesis in breast cancer. There is preclinical and early clinical evidence that agents targeting the Notch pathway, of which the GSIs are most advanced, may be effective in this disease. Further clinical trials will hopefully confirm the efficacy of Notch pathway inhibition either as a single agent or in combination with endocrine therapy, targeted therapies, chemotherapy, or possibly even radiation therapy as novel approaches, ultimately leading to improved patient outcomes overall.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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