Abstract: The numerous processes involved in the etiology of breast cancer such as cell survival, metabolism, proliferation, differentiation, and angiogenesis are currently being elucidated. However, underlying mechanisms that drive breast cancer progression and drug resistance are still poorly understood. As we discuss here in detail, the Notch signaling pathway is an important regulatory component of normal breast development, cell fate of normal breast stem cells, and proliferation and survival of breast cancer initiating cells. Notch exerts a wide range of critical effects through a canonical pathway where it is expressed as a type I membrane precursor heterodimer followed by at least two subsequent cleavages induced by ligand engagement to ultimately release an intracellular form to function as a transcriptional activator. Notch and its ligands are overexpressed in breast cancer, and one method of effectively blocking Notch activity is preventing its cleavage at the cell surface with γ-secretase inhibitors. In the context of Notch signaling, the application of clinically relevant anti-Notch drugs in treatment regimens may contribute to novel therapeutic interventions and promote more effective clinical response in women with breast cancer.

Keywords: breast cancer, signaling pathways, γ-secretase, γ-secretase inhibitors, combination breast cancer therapy

Introduction

In recent years, there have been many advances in deciphering critical cell signaling networks and their relationship to the driving forces of cancer onset, growth, and metastasis. Moreover, in the hierarchy of signaling pathways, several pathways are considered fundamental to regulation of cell fate and having widespread survival effects, namely the Notch, Wnt/Wingless (Wnt), and Hedgehog (HH) pathways. This review will focus on the role of the canonical Notch signaling pathway in breast cancer etiology and progression. Furthermore, we will review the current therapeutic options available for inhibiting Notch. Blockade of an upregulated Notch signaling pathway can be achieved by inhibiting the formation of the main force of Notch activity, the Notch intracellular domain (NICD). Thus, a pharmacological approach using γ-secretase inhibitors (GSIs) to prevent the final cleavage step of the precursor form of Notch, ie, transmembrane Notch (Notch™) that will decrease levels of NICD could be a novel therapeutic strategy either as a single agent or in combination with targeted or cytotoxic chemotherapy for a subset of cancer patients.

Breast cancer subtypes

Breast cancer is a heterogeneous disease divided into four major subtypes: luminal A (estrogen receptor [ER]+/progesterone receptor [PR]+), luminal B (ER+/PR+/human...
epidermal growth factor receptor [HER]-2+, HER-2+/neu+, and triple negative (ER-PR-/HER-2+). Breast cancer of the luminal A or B subtype is derived from the luminal epithelium of the breast ducts, and these tumors express hormone receptors, ER and PR. These subtypes comprise 70%–80% of all breast cancers. The ER+/PR+ luminal A subtype is very sensitive to current antihormonal therapy such as tamoxifen, fulvestrant, or aromatase inhibitors. Luminal B breast tumors have a higher proliferative index than those of luminal A and are inherently more resistant to current antihormonal therapy. HER-2+/neu+ designates a breast cancer subtype that contains gene amplification for the ERBB2 proto-oncogene resulting in overexpression of the HER-2 receptor tyrosine kinase protein. The HER-2+/neu+ breast cancers are very sensitive to anti-HER-2 therapy such as trastuzumab or lapatinib. The final subtype of breast cancer is triple negative, which lacks expression of ER, PR, and HER-2. Triple negative breast tumors are the most aggressive, with poor prognosis and currently no approved targeted therapy. These triple negative breast tumors are treated with cytotoxic chemotherapy such as a DNA-damaging agent (cis- or carboplatin) or tubulin-destabilizing compounds (taxanes).

Although dramatic improvements have been made to cure breast cancer, one of the major problems that continue to plague both research scientists and clinicians is drug resistance. Therefore, elucidating the critical mechanisms that contribute to drug-resistant breast cancer will hopefully prevent tumor recurrence and disease progression and ultimately provide a “cure” to women with breast cancer.

**Notch signaling**

Over a century of research has revealed the mechanisms that regulate canonical Notch signaling in the context of cell-to-cell signaling that controls both embryonic and adult stem cell self-renewal, stem cell quiescence, cell fate and differentiation, cell survival, apoptosis, and tumorigenesis. Investigations elucidating the Notch pathway date back to the early 20th century, when in 1913 John Smith Dexter working in the laboratory of American geneticist Thomas Hunt Morgan observed the outcome of a mutation of a gene in Drosophila ampedelophila, which resulted in a notch or indentation at the ends of the fly wings. He called them “perfect notched.” Additional research in 1917 by Morgan identified the alleles of this fly gene which eventually became known as “Notch” and he published his findings in *The Physical Basis of Heredity* in 1919. The Notch gene was eventually cloned and identified for the first time in 1985–1986. Related research employing the nematode worm *Caenorhabditis elegans* further elucidated the Notch signaling pathway, cell-to-cell interactions, and lateral inhibition during embryogenesis. Presently, embryologists and cancer researchers are the largest groups of research scientists studying Notch signaling.

**Developmental Notch**

The Notch signaling pathway mediates cell fate determination in three ways: regulatory, inhibitory, and inductive action. In regulatory signaling, during embryogenesis, Notch regulates the development and differentiation of many organ systems (angiogenesis, hematopoiesis, homeostasis, neurogenesis, nephrogenesis, myogenesis, and somatogenesis). The importance of this function was verified in Notch-1, Notch-2, Jagged-2, and Delta-1 knockout mice, which lack each Notch receptor or ligand regulatory components. The mice exhibited severe defects which resulted in embryonic or perinatal death. Notch activation in pluripotent stem cells initiates lateral inhibition so that a certain number of cells take on a specific cell fate and those adjacent are inhibited from differentiating. This process is exemplified in the following experiment: nascent chick retinal neurons transiently overexpressing Deltex-1 were found to prevent adjacent neuroepithelial progenitor cells from differentiating into neurons. Lastly, in inductive signaling, Notch promotes or induces the development of a certain cell type, usually amongst different (nonequivalent) cells. Such interactions are important for establishing demarcated boundaries between cell types, and the signaling is aptly referred to as boundary formation. The necessity of inductive signaling is evident in developmental studies. For example, a Notch-dependent localized signal affected the formation of the dorsoventral wing organizer in *Drosophila*, while the expression of Radical fringe determined the position of the dorsoventral boundary of vertebrate limbs.

**Notch receptors**

The Notch receptor is classified as a large single-pass type 1 transmembrane glycoprotein. It is expressed as a heterodimer at the cell membrane. There are four mammalian Notch receptors, Notch 1–4, with Notch-1 being the longest and Notch-4 the shortest. They are comprised of three domains, extracellular, transmembrane, and intracellular (Figure 1). Notch is synthesized as a single, relatively large (>300 kDa) polypeptide in the endoplasmic reticulum. There, it undergoes O-glycosylation. An initial addition of O-linked fucose to the epidermal growth factor (EGF)-like repeats is mediated by O-fucosyl transferase 1. After the Notch preprotein is chaperoned by the guanosine triphosphate hydrolase...
Notch receptors and ligands.

In vertebrates, the Notch ligands are known as Delta-like 1, 3, and 4 and Jagged-1 and 2 (Figure 1). They are single-pass Type 1 transmembrane proteins that bind and activate the Notch receptor “in trans” (at the surface of a neighboring cell). They have extracellular and intracellular domains. The Jagged ligands are longer than the Delta-like ligands, the length determined by the 6–16 EGF-like repeats in the extracellular domain. A cysteine-rich area is located at the end of the EGF-like repeats, with Jagged ligands having a longer cysteine-rich area compared to the Delta-like ligands.

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Notch receptor in a nonactivated state. The third part of the Notch receptor is the intracellular domain (C-terminus), which extends from the inner cell membrane into the cytoplasm. It contains four separate entities: (1) the DNA-binding protein recombination signal-binding protein for immunoglobulin kappa J (RBP-JK associated molecule or RAM domain), followed by a linker with a nuclear localization sequence; (2) seven iterated cdc10/ankyrin repeats; (3) a transcription activation domain (TAD) with an additional nuclear localization sequence; and (4) polypeptide proline, glutamate, serine, and threonine-rich motifs (PEST) with degradation signals or degrons that stabilize NICD in the nucleus and target it for rapid proteolytic degradation. Lastly, TAD is found in Notch-1 and Notch-2, but not in Notch-3 and Notch-4.

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As reviewed in (GTPase) Rab-protein 6 through the secretory pathway to the trans-Golgi network, it undergoes additional elongation of the O-fucose with carbohydrate chains on serine and threonine residues by the Fringe family O-fucose-specific β1,3-N acetylglicosaminyl-transferases Lunatic, Manic, or Radical. Modification of the Notch receptor by Fringe proteins controls ligand-mediated activation. Next is cleavage by furin-like convertase into the N-terminus and C-terminus subunits and subsequent translocation of these mature entities to the cell plasma membrane. There, the cleaved subunits are assembled into the cell membrane as a fully functional heterodimeric receptor, noncovalently linked by a calcium cation awaiting engagement with a Notch ligand.

The N-terminal extracellular domain of each Notch receptor is the ligand-binding component and consists of 29–36 multiple EGF-like repeats in tandem. From each extracellular domain extend six cysteine residues, which form three intra-domain disulfide bridges. Adjacent to the extracellular domain and closer to the cell membrane is the transmembrane domain, a dual hybrid moiety. The two components of this domain are: (1) the juxtamembrane RAM23 section (the negative regulatory region) made of three Lin-12/Notch repeats, which prevent ligand-independent interactions, plus two conserved cysteine residues; and (2) the heterodimerization section, which maintains the Notch receptor in a nonactivated state. The third part of the Notch receptor is the intracellular domain (C-terminus), which extends from the inner cell membrane into the cytoplasm. It contains four separate entities: (1) the DNA-binding protein recombination signal-binding protein for immunoglobulin kappa J (RBP-JK associated molecule or RAM domain), followed by a linker with a nuclear localization sequence; (2) seven iterated cdc10/ankyrin repeats; (3) a transcription activation domain (TAD) with an additional nuclear localization sequence; and (4) polypeptide proline, glutamate, serine, and threonine-rich motifs (PEST) with degradation signals or degrons that stabilize NICD in the nucleus and target it for rapid proteolytic degradation. Lastly, TAD is found in Notch-1 and Notch-2, but not in Notch-3 and Notch-4.

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an additional cysteine-rich area. The intracellular domain of each ligand has a shorter cytoplasmic tail than the extracellular domain and contains a PDZ (post synaptic density protein [PSD95], Drosophila disc large tumor suppressor [Dlg1], and zona occludens-1 protein [zo-1])-binding motif which aids in intracellular protein–protein interactions. The ligand-activated cell-surface receptor initiates a cascade of events with two subsequent proteolytic cleavages that result in NICD entry into the nucleus to function as a transcriptional activator.\textsuperscript{27–29}

Cell-cell contact mediates Notch ligand to receptor binding which initiates short-range cell-to-cell communication, a mono-directional cascade of events beginning at the cell membrane and ultimately activating the CSL (C promoter binding factor-1 [CBF-1], suppressor of hairless, Lag-1) family of transcription factors in the nucleus. The ligand engages the Notch receptor through its cognate high affinity EGF-like repeats (Figure 2). Ligand-mediated endocytosis in the ligand-expressing cell (trans-endocytosis) provides a force to pull the extracellular domain of the Notch receptor from the transmembrane domain. This mechanical pull exposes the S2 cleavage site for the \( \gamma \)-secretase to excise the Notch intracellular domain (NICD) from the inside of the cell membrane, allowing it to enter the cytoplasm and ultimately activate the CSL (C promoter binding factor-1) family of transcription factors in the nucleus. The ligand-expressing signal-sending cell, followed by endosomal-mediated degradation or recycling. Monoubiquitination by E3 ligases Mindbomb-1 and -2 or Neuralized-1 and -2 marks the ligand for endocytosis.

The remaining NEXT portion now exposes the S3 and S4 cleavage sites that are mediated by the \( \gamma \)-secretase complex.\textsuperscript{31} Interestingly, there are many \( \gamma \)-secretase substrates, a great number having relevance in breast cancer.\textsuperscript{34,35} This transmembrane aspartyl proteinase, considered a large complex, is comprised of a catalytic subunit designated presenilin 1 or presenilin 2, a seven-pass transmembrane protein, and accessory subunits comprised of the transmembrane proteins nicastrin (NCT), anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN-2), a two-pass transmembrane protein. Nicastrin and APH1 stabilize PEN-2, which induces endoproteolysis of presenilin.\textsuperscript{36} Following receptor activation, NICD that is still attached to the inner cell membrane is marked for proteosomal degradation by E3 ubiquitin ligases Numb and Itch. \( \gamma \)-secretase severs NICD from the inside of the cell membrane, allowing it to enter the cytoplasm\textsuperscript{37} and eventually translocate to the nucleus (Figure 2).\textsuperscript{38}

**Figure 2** Significant components in the Notch signaling pathway.

Abbreviations: ADAM/TACE, a disintegrin and metalloprotease/TNF-\( \alpha \)-converting enzyme; TNF-\( \alpha \), tumor necrosis factor-alpha; APH1, anterior pharynx-defective 1; ER, endoplasmic reticulum; NEXT, Notch extracellular truncation; NICD, Notch intracellular domain; PEN-2, presenilin enhancer 2; EGF, epidermal growth factor; S, site.
NICD forms a transcriptional activation complex with CSL in the nucleus once the ankyrin-repeat motif of NICD docks with the Rel homology region of the DNA-binding factor CSL. Thus, CSL changes from a transcriptional repressor to a transcriptional activator. There occurs a release of transcription factor co-repressors (CoRs) like class I or II histone deacetylases, CBF-1-interacting repressor (CIR), SKI-interacting protein (SKIP), silencing mediator of retinoid and thyroid hormone receptor (SMRT), and SMRT/HDAC (histone deacetylase)-1-associated repressor protein (SHARP), and a recruitment of transcription factor co-activators (CoAs) such as mastermind-like 1–3 (MAML) protein. MAML further recruits the histone acetyltransferases, cyclic AMP (adenosine monophosphate) response element-binding (CREB) protein CBP/p300 and p300/CBP-associated factor or general control non-depressible 5 (GCN5), to acetylate histone tails for the unwinding of nucleosomes within chromatin for active transcription. This leads to an increased expression of specific genes. Some of the Notch gene targets that can be activated are: c-Myc, p21, and cyclin D1 (cell cycle progression), Bcl-2 (inhibition of apoptosis), and hairy and enhancer of split basic helix-loop-helix HES 1, 5, 6, and 7, and HEY 1 and 2, and HEY-L family of proteins (transcriptional repressors). NICD activity in the nucleus ends with phosphorylation triggered by cyclin c-cyclin-dependent kinase 8 (C-CDK8). Subsequently, glycogen synthase kinase 3β phosphorylates the PEST domain of the C-terminus of the NICD, which is then targeted for polyubiquitination by E3 ligase SEL10/FWB7 in the proteosome. Figure 3 depicts Notch-mediated nuclear transcription.

**Notch and cancer: general overview**

One of the earliest associations between Notch signaling and cancer occurred in 1991 in human T-cell acute lymphoblastic leukemia, where the Notch-1 gene was associated with the t(7;9)(q34;q34.3) chromosomal translocation. Notch cell signaling defects were detected in the form of alterations in the Notch-1 negative regulatory region and a loss of the C-terminus PEST domain, both of which lead to increased Notch-1 intracellular domain (N-1ICD) activity. In B-cell malignancies such as chronic lymphocytic leukemia, Notch-1 mutations were linked to increased disease progression and resistance to chemotherapy. Inconsistencies in the role of Notch in malignant B-cells became apparent as some data indicated that Notch signaling inhibited B-cell growth, while other data reported a Notch-induced increase in B-cell proliferation. In mantle cell lymphoma, Notch-1 or Notch-2 PEST domain mutations were reported. In addition to the presence of dysfunctional Notch receptors in leukemia, the ligand Jagged-2 was found to be significantly overexpressed in multiple myeloma. In addition to hematologic malignancies, aberrant Notch signaling has been found in solid tumors;
for example, cervical,\textsuperscript{72,73} colon,\textsuperscript{54,55} liver,\textsuperscript{56,57} lung,\textsuperscript{58} pancreatic,\textsuperscript{59–62} prostate,\textsuperscript{63,64} ovarian,\textsuperscript{65,66} and renal.\textsuperscript{67} Indeed, based on the numerous reports on the role of Notch signaling in cancer development and progression, Notch signaling has become a major target for novel therapeutic strategies.\textsuperscript{58–72} The role of Notch signaling in cancer could possibly be a double-edge sword. It was reported that Notch receptors and ligands were both oncogenic and tumor-suppressive in the same tumor.\textsuperscript{73} The possibility that Notch promotes or suppresses tumor growth has also been put forth by others.\textsuperscript{74–76} Some discrepancies in Notch signaling in cancer may be explained in part by “cell context, dose, and timing,”\textsuperscript{77} as well as Notch cross-talk with other signaling pathways, the micro-tumor environment, and the stage of cancer at the time of detection.

**Notch and breast cancer**

There is strong evidence that Notch signaling is dysregulated in solid tumors,\textsuperscript{28,76,78} though as reported in leukemia, it may be both a tumor oncogene and suppressor in breast and other cancers.\textsuperscript{79} In mouse studies, tissue specific expression of N-1ICD induces spontaneous mammary tumors.\textsuperscript{80–82} Furthermore, transgenic (Tg) mice expressing mammary specific N-4ICD also form spontaneous mammary tumors.\textsuperscript{83} In fact, Notch-1 and Notch-4 are categorized as bonafide breast oncogenes.\textsuperscript{84} Further studies showed that overexpression of Notch-2\textsuperscript{85} and Notch-4\textsuperscript{86–88} also leads to murine mammary tumor formation. Studies from human breast cancer cell lines show deregulated expression of Notch and Notch ligand messenger RNA (mRNA).\textsuperscript{88} Results from a human xenograft model for inflammatory breast carcinoma (MARY-X) implicate altered Notch-3 signaling specifically.\textsuperscript{89} In a study of 200 Greek women from differing breast cancer subtypes, Notch-4 mRNA levels were found to be higher in the hormone receptor and HER-2-positive breast cancers, while Notch-1 and Notch-3 mRNA levels were higher in triple-negative specimens compared with normal tissue.\textsuperscript{90} When Notch-1 and Notch-4 and Jagged-1 and Delta-like-1 expression were measured by immunohistochemistry in breast hyperplasia and carcinomas, high levels of Notch-1 were found in the hyperplasias, ductal carcinoma in situ, infiltrating ductal carcinomas (IDCs), and infiltrating lobular carcinomas (ILCs), as well as elevated expression of Notch-4 and Jagged-1 in IDCs and ILCs.\textsuperscript{91} Moreover, Notch-1 and Notch-3 NICD levels were increased in both human breast cancer specimens and cell lines, and Notch-3 activated nuclear transcription in those specimens and cells.\textsuperscript{92} Further evidence for altered Notch-1 in human breast cancer was found in the form of aberrant Notch-1 activation in various breast cancer subtypes.\textsuperscript{93} In addition, samples from breast cancer patients showed co-overexpression of the Notch-1 receptor and its ligand Jagged-1 predicting the poorest patient survival.\textsuperscript{94,95} Lastly, examination of almost 100 breast cancer specimens by immunohistochemistry and quantitative polymerase chain reaction (PCR) showed the expression of Notch-1 also correlated with poor outcomes.\textsuperscript{96}

Nonetheless, Notch receptors are not a homogeneous group functionally. When the transcriptional activities of N-1ICD, N-2ICD, and N-3ICD on HES-1 and HES-5 promoters were measured using a luciferase reporter assay, some of the differences were related to the combination of receptors used and expression level of RBP-Jk (CSL or CBF-1). Also, inhibitory Notch-2 activity was confirmed, as co-expression of N-2ICD with N-1ICD or N-3ICD reduced their activity.\textsuperscript{97} In a xenograft study using MDA-MB-231 cells, Notch-2 inhibited tumor growth.\textsuperscript{98} Similarly, a clinical study which examined Notch-2 expression in breast cancer tissue by immunohistochemistry and qualitative and quantitative PCR concluded it may function as a tumor suppressor.\textsuperscript{96}

In assessing the role of Notch signaling in breast cancer stem cells, it was concluded from in vitro and in vivo experiments that Notch-4 activity was eightfold higher in breast cancer stem cells than in differentiated cells, and inhibition of Notch-4 resulted in suppression of tumor growth.\textsuperscript{99} Moreover, breast cancer stem cells exhibited increased Notch signaling as compared with bulk tumor cells, especially in levels of HES-1 mRNA, and GSIs effectively blocked mammosphere formation, which is an assay to measure survival and self-renewal of breast cancer stem cells.\textsuperscript{100}

**Notch and tumorigenesis**

Of the more than 300 breast cancer cases examined, approximately 50% showed a loss of Numb-mediated inhibition of Notch signaling by ubiquitination and proteosomal degradation.\textsuperscript{101} Of particular interest are two germline alterations (R62H and R71W) of presenilin-2 (PS-2) that have been reported in breast cancer patients with axillary node-negative disease, resulting in PS-2 being more susceptible to degradation.\textsuperscript{102} Furthermore, nicastrin-knockout mice, which have decreased proteolytic cleavage of Notch and consequently lower NICD, developed myeloproliferative disease,\textsuperscript{103} and **Notch-1 knockout mice formed spontaneous basal cell carcinoma as they grew older.**\textsuperscript{104}

Possible mechanisms of action for Notch-driven tumor propagation are: gain of function mutation, ligand-mediated activation of Notch, and downregulation of Notch.\textsuperscript{105} Nonetheless, Notch tumorigenicity may be organ-dependent. In self-renewing systems such as skin, intestine, and bone
marrow, Notch interacts with multiple signaling pathways. Oncogenesis derails these interactions such that Notch becomes a tumor suppressor in the skin and an oncogene in the bone marrow.\(^7\) Manipulation of gene expression has been useful to study Notch receptors and ligands in tumorigenic systems. For example, MCF-10A cells, considered nonmalignant and noninvasive, when transfected with Notch-4, grew in a soft agar assay, suggesting that Notch-4 is a breast oncogene.\(^10\) Similarly, mice bred to express Notch-1ICD and Notch-3ICD in mammary epithelial cells developed mammary tumors.\(^3\) Nonetheless, Notch receptors may not be equivalent in their capacity to induce cancer. Notch-2 may suppress tumorigenicity, as MDA-MB-231 cells with constitutively expressing N-2ICD showed increased apoptosis and did not form xenograft tumors in mice.\(^9\) Notch signaling is also responsive to hormonal drivers of tumorigenicity, since estrogen was found to upregulate Notch-1 and Jagged-1 in MCF-7 cells.\(^7\) In contrast, Rizzo et al demonstrated that estrogen-mediated ER activation suppresses Notch activation, and the combination of anti-estrogen therapy with a GSI was more effective in inhibiting ER+ breast tumor growth than either therapy alone.\(^11\) Specifically, the same group identified that Notch-1ICD activates ER-responsive genes under low estrogen conditions, suggesting that Notch-1ICD could mediate activation of the ER in an estrogen-independent manner.\(^7\)

Furthermore, loss of negative regulatory mechanisms contributes to neoplastic metastasis. For example, expression levels of the negative regulator of Notch signaling Numb inversely correlated with tumor aggressiveness.\(^10\) In in-vitro and in-vivo experiments examining osteolytic bone metastasis of human breast cancer cells, osteoblasts together with secretion of transforming growth factor β1 enhanced Notch-3 expression in the breast cancer cells and mediated their metastasis; this effect was inhibited by GSI L-685458.\(^8\)

### Notch and oncogenic crosstalk

The oncogenic reach of the Notch signaling pathway is partly due to its communication or crosstalk with other signaling pathways. Hurlbut et al\(^10\) proposed more than 50 connections for the Notch crosstalk network; for example, receptor tyrosine kinases (RTKs), HH, Janus kinase/signal transducers and activators of transcription (Jak/STAT), transforming growth factor-β/decapentaplegic (TGF-β), and Wnt pathways. In addition to HH, Wnt, and TGF-β, amongst others pertinent to Notch crosstalk are platelet-derived growth factor (PDGF/PDGFR), vascular endothelial growth factor (VEGF), phosphatidylinositol 3-kinase (PI3K/Akt), Ras, mammalian target of rapamycin (mTOR), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), hypoxia-inducible factor (HIF), and cytokines interleukin-6 (IL-6), IL-1, and leptin plus ER signaling as well as microRNAs considered operationally important for Notch crosstalk in breast cancer.\(^11\) The majority of functions which include cell proliferation, differentiation, and development, tumor angiogenesis, morphogenesis, and somitogenesis are all important during oncogenesis. One of the most critical pathways necessary for survival of cancer cells is the NF-kB pathway. It has been shown that NF-κB regulates Notch and is regulated by Notch. For example, N-1ICD or N-3ICD has been shown to activate NF-kB signaling components such as IKK (inhibitor of kappa B kinase).\(^12\) Furthermore, NF-κB has also been shown to regulate Notch indirectly by inducing Jagged-1, HES-5, and/or Deltex-1.\(^13\)

The existence of Notch and RTK crosstalk in breast and other solid tumors has been established by our research group and many others since. We and others have shown that Notch-1 signaling is decreased in ErbB2 overexpressing BT-474, SkBr3, and MCF-7/HER2 breast cancer cells and that anti-HER-2 therapy using trastuzumab or a small molecule tyrosine kinase inhibitor similar to lapatinib reactivated Notch-1. More importantly, a GSI or specifically Notch-1 knockdown increased the sensitivity of ErbB-2+ breast cancer cells to anti-HER-2- mediated growth inhibition, indicating that Notch-1 signaling might contribute to trastuzumab resistance in vitro.\(^14\) Moreover, tumor recurrence was prevented in mice injected with trastuzumab-sensitive BT-474 cells following treatment with trastuzumab and MRK-003 GSI or significantly reduced with trastuzumab and LY-411575 GSI; additionally, BT-474 breast tumors that were resistant to trastuzumab were re-sensitized by addition of MRK-003 GSI.\(^15\) An overview of the role and significance of Notch signaling in trastuzumab resistant breast cancer is reviewed by Mehta and Osipo.\(^16\)

The regulation and activation of Notch signaling in triple negative breast cancer was recently elucidated by Clementz et al.\(^17\) Specifically, the investigators demonstrated that PEA3, an Ets family transcription factor, activates transcription of Notch-1 and Notch-4.\(^17\) It was identified that enrichment of PEA3 on the Notch-1 promoter was independent of AP-1 while PEA3 recruitment to the Notch-4 promoter was dependent on c-JUN and Fra-1, but negatively regulated by c-Fos. The findings from this study also showed that knockdown of PEA3 was potent in inhibiting triple negative breast cancer growth in vitro and in vivo.
**Table 1 Chemical structure of γ-secretase inhibitors**

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<td>Deshydroxy LY-411575</td>
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<td></td>
<td>Compound E</td>
<td><img src="image" alt="Compound E Structure" /></td>
<td>Non-transition state analog</td>
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<td>GSI-XXI</td>
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<tr>
<td></td>
<td>RO-4929097 (Roche, Nutley, NJ, USA)</td>
<td><img src="image" alt="RO-4929097 Structure" /></td>
<td>Transition state analog</td>
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<tr>
<td>Sulfonamide</td>
<td>MRK-003 (Merck and Co, Boston, MA, USA)</td>
<td><img src="image" alt="MRK-003 Structure" /></td>
<td>Non-transition state analog</td>
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<tr>
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<td>MK-0752 (Merck and Co)</td>
<td><img src="image" alt="MK-0752 Structure" /></td>
<td>Non-transition state analog</td>
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<tr>
<td>Selective</td>
<td>PF-03084014 (Pfizer Inc., Groton, CT, USA)</td>
<td><img src="image" alt="PF-03084014 Structure" /></td>
<td>Non-transition state analog</td>
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**Abbreviations:** GSI, gamma-secretase inhibitor; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

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**GSIs: mode of action and side effects**

The more than 100 GSIs synthesized to date can be divided into three classes: peptide isosteres, azepines, and sulfonamides. They are oral agents, the azepines and sulfonamides being the most popular. A list of select GSIs is presented in Table 1. GSIs currently undergoing US clinical trials are listed in Table 2.

The GSIs are classified into two types, depending on structure and binding sites: (1) aspartyl proteinase transition-state analogs as peptide isosteres that mimic the transition state of...
a substrate cleavage by \( \gamma \)-secretase and bind competitively to the catalytic active site of presenilins, and (2) small molecule non-transition-state inhibitors where the binding site is different from the active site, possibly at the interface of the \( \gamma \)-secretase complex dimer. \(^{120}\) Well known side effects of GSIs occur within the gastrointestinal tract. For example, acute treatment of TgCRND8 mice with LY-411575 for 15 days caused an increase in the number of mucin-containing goblet cells in the small and large intestines and changes in the tissue architecture of the gastrointestinal tract which resulted in severe diarrhea. \(^{121}\) The GSIs also cause various off-target effects in breast cancer cells and Notch signaling. An early transition-state analog GSI, IL X (cbz-IL-CHO), produced a decrease in mRNA and protein levels of HES-1, induced G0/G1 cell cycle arrest, and inhibited human tongue carcinoma Tca8113 cell growth. \(^{122}\) Dipeptide GSI XII (z-Leu-Leu-CHO) induced apoptosis in breast cancer cell lines by inducing Noxa, a pro-apoptotic protein. \(^{123}\) A later generation GSI, LY-294002 suppressed angiogenesis by blocking the epidermal growth factor (EGF)-induced upregulation of Jagged-1 in squamous cell carcinoma, thereby inhibiting EFG-Notch crosstalk. \(^{134}\) Tripeptide GSI I (z-Leu-Leu-Nle-CHO) suppressed cell proliferation and induced apoptosis in Notch-3 overexpressing ovarian cell lines. \(^{65}\)

An early generation non-transition state analog is DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, a dipeptide inhibitor of the benzodiazepine type, also known as GSI IX and Compound 3. \(^{125}\) It is the most widely used in the laboratory setting. \(^{126}\) DAPT potentiates the apoptotic effects of the DNA-damaging drug melphalan in MCF-7 breast cancer cells. \(^{127}\) In cell lines with chromosomal translocations, DAPT inhibited the proliferation of truncated Notch-1 expressing an ADAM cleavage site but not of truncated Notch-2 which was without the cleavage site. \(^{128}\) From DAPT, numerous other GSIs have been developed that are even more effective, ie, LY-411,575 (Compound 5) 100-fold stronger than DAPT, LY-450,139 (Semagacestat or Compound 6), \(^{129}\) and RO-4929097 (Roche, Nutley, NJ, USA).

The small-molecule GSI classified as a tetralin imidazole PF-03084014 (Pfizer Inc., Groton, CT, USA) is in a Phase I trial to treat advanced breast cancer and other solid tumors. \(^{130}\) It is considered a selective or Notch-sparing GSI or GS (gamma secretase) modulator. When evaluated for Notch activity, PF-03084014 significantly decreased tumor cell migration and mammosphere formation in vitro, reduced tumor cell self-renewal ability in vivo, and decreased mRNA expression of Notch target genes HES-1, HES-4, Notch-1, and HEY-2 in HCC1599 xenograft tumors. \(^{131}\)

Another small-molecule GSI, RO-4929097, was used in a multicenter Phase I clinical dose escalating study and continued on to Phase II and combination therapy studies. Derived from LY-411575 and containing a dibenzazepinone core, it is being tested for the treatment of breast cancer and other solid tumors. \(^{132}\) Patients with low basal levels of plasma IL-6 and IL-8 responded well, indicating that cytokines may be predictive biomarkers for response to therapy. \(^{134}\) In in vitro studies using a colon cancer cell line A549, RO-4929097 produced a significant decrease in mRNA levels of Notch target genes HES-1, HES-4, and HEY-1. \(^{135}\)

Another GSI, the sulfonamide-containing non-transition-state GSI analog MK-0752 (Merck and Co, Inc, Whitehouse Station, NJ, USA)
Station, NJ, USA) is in a Phase I study to treat metastatic or locally advanced breast cancer.

**Novel combination strategies**

Since single-drug therapy is ineffective for long-term use, combination therapy oftentimes becomes necessary. Such a treatment regimen is applicable to “endocrine therapy, targeted therapies, chemotherapy, or possibly even radiation therapy.”

There are several clinical studies using RO-4929097 in combination therapy. One Phase I study is using RO-4929097 with capcetabine for patients with refractory solid tumors and another with paclitaxel and carboplatin for patients with Stage I or III triple negative breast cancer. A Phase I clinical trial is presently underway testing the efficacy of RO-4929097 and a potent HH antagonist GDC-0449 vismodegib in patients with advanced breast cancer. These patients may have been selected on the basis of upregulated crosstalk between Notch and the self-renewal pathway (targeted therapy). Another Phase I study with RO-4929097 is adding cediranib for post-menopausal patients with advanced solid tumors (targeted therapy). In a Phase Ib clinical trial, RO-4929097 is combined with letrozole for patients with ER+/PR+ Stage II or III breast cancer (endocrine therapy). Another GSI in combination therapy is MK-0752. In a Pilot study, MK-0752 is being combined with tamoxifen or letrozole for patients with early stage breast cancer (endocrine therapy). In a Phase I study, MK-0752 (or MK-2206 Akt inhibitor) is being combined with ridaforolimus (MK-8669) in patients with advanced solid tumors (targeted therapy). A Phase I/II study is combining MK-0752 with docetaxel in patients presenting with locally advanced or metastatic breast cancer (chemotherapy).

Gastrointestinal toxicity is a major side-effect with GSI use. Nonetheless, careful monitoring of treatment protocols, whether by modulating expression of Notch receptors with receptor antibody pretreatment before GSI treatment or development of a practical combination therapy should minimize problematic side-effects. Notch activation must be assessed prior to GSI treatment (mutations and/or overexpression), since GSIs are more effective against tumors with upregulated Notch signaling. In addition, close attention must be paid to the therapeutic window so that the minimally active dose needed to inhibit Notch is employed, thereby reducing adverse side effects.

**Conclusion**

Much progress has been made in understanding Notch signaling in breast cancer. Molecular profiling of patients, fast becoming standard of care, identifies the type and location of signaling dysfunction. Moreover, pharmacological innovations are helping produce more selective GSIs with fewer side effects. A “one problem–one solution” type of care to breast cancer seems unlikely. Inhibition of Notch signaling with pharmacodynamically active drugs such as the GSIs is preventing metastasis and recurrence and increasing disease-free survival. The next level of care for determining the molecular signature of a breast tumor will develop therapeutic combinatorial protocols that effectively target crosstalk pathways, tumor microenvironment, tumor-initiating cells (or cancer stem cells), developmental factors, non-canonical signaling components, and possibly other additional modulating factors still unknown. Breast cancer management will require a multidisciplinary team to prepare and optimize the anticancer drug regimen, conduct the therapy, and even interpret results and treatment progress. Overall, targeting the Notch signaling pathway in breast cancer therapy and attempting its downregulation with GSIs looks promising.

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**Disclosure**

The authors report no conflicts of interest in this work.

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