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Notch Antagonists: Potential Modulators of Cancer and Inflammatory Diseases

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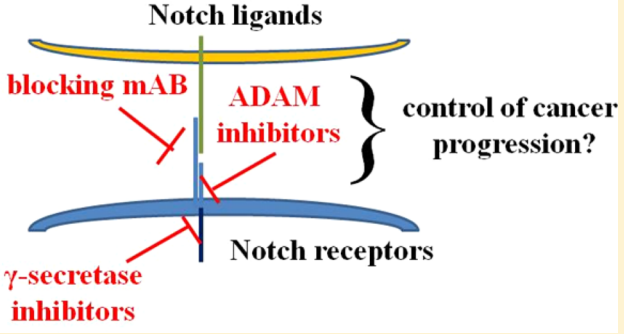
ABSTRACT: Notch is a key player in various developmental processes during the embryonic stage as well as in regulating tissue homeostasis, cell differentiation, and stem cell maintenance in adult life. Activation of Notch signaling occurs following Notch receptor−ligand interaction and subsequent enzymatic proteolysis by the gamma-secretase complex, resulting in the cytoplasmic release of a Notch intracellular domain, which translocates to the nucleus to initiate the downstream transcriptional machinery. Notch activation and its aberrant signaling have been broadly linked to the pathogenesis of cancer and some chronic inflammatory diseases resulting in pathologic fibrotic processes. This review focuses on the molecular basis of Notch-induced signaling and its interaction with other pathways to identify therapeutic targets. We also highlight current efforts to pharmacologically intervene in Notch signaling and discuss promising ongoing experimental and clinical studies.



1. INTRODUCTION

Notch signaling was first described a century ago after the discovery of notched (toothed) wings in the fruit fly *Drosophila melanogaster* resulting from a sex-linked mutation in the gene later known as *Notch*.[1] Notch signaling plays a critical role in many fundamental processes, including cell proliferation, apoptosis, activation of differentiation programs, and specific cell fates. The aberrant gain or loss of function of Notch signaling components has been related to many human diseases. The first evidence for the involvement of Notch genes and receptor precursors in cancer was reported in T-cell acute lymphoblastic leukemia (T-ALL).[2] However, altered signaling of the Notch pathway has been linked to numerous diseases, including various cancers, fibrosis, and degenerative diseases. Notch receptors are type I transmembrane proteins. To be activated following the translation of the Notch genes, Notch receptor precursors need several steps of glycosylation and an intracellular proteolytic step to be expressed at the cell surface. Then, following engagement with their cognate ligands, two sequential proteolytic steps release the active intracellular domains of the receptors, which then migrate toward the nucleus to perform their transcriptional activity. In particular, the proteolytic steps, involving serine- (furins), metallo- (a disintegrin and metalloproteinase, ADAMs) and aspartyl (γ-secretase) proteases, are necessary to activate the Notch proteins. These proteolytic activities are widely expressed in normal and diseased tissues, and their activities are essential to modulate the functions of other biological peptides required for the normal homeostasis of cells, tissues, and organs, as well as in diseases. Thus, for selective therapy, it is mandatory to achieve only localized inhibition of these activities to protect the other functions of these enzymes and also to maintain essential Notch-dependent signaling pathways in nontarget tissues. The strategies used initially have been nonspecific and associated with detrimental side-effects. More recently, improved selectivity and decreased toxicity have been sought. The design of new inhibitors of γ-secretase to achieve blockade of the Notch pathway over other pathways, or the development of antibodies directed either against the Notch receptors or Notch ligands, are some examples of these recent attempts at selective and specific control of Notch. In this perspective, we provide a brief overview of the importance of Notch signaling in cellular biology by describing the main components of Notch signaling, focusing on the unique features of this pathway in some pathological conditions. Apart from that we also highlight potential targets for pharmacological interventions and some therapeutic strategies to control Notch functions.

1.1. Notch Ligand−Receptor Interaction. *Canonical Notch Signaling.* Notch signaling is primarily induced by binding of specific ligands from the Delta-like (DLL-1, 3, and 4) and Jagged (Jagged 1 and 2) families (Figure 1).[3,4] Notch ligands and their receptors (Notch 1−4) are transmembrane proteins with extracellular domains that possess varying numbers of epidermal growth factor (EGF)-like repeats. The Notch-Delta signaling pathway was first described as being involved in communication between neighboring cells during development. Delta ligands were reported to have two different activities: Delta in one cell can bind and transactivate Notch signaling in a neighboring cell while inhibiting this signaling in

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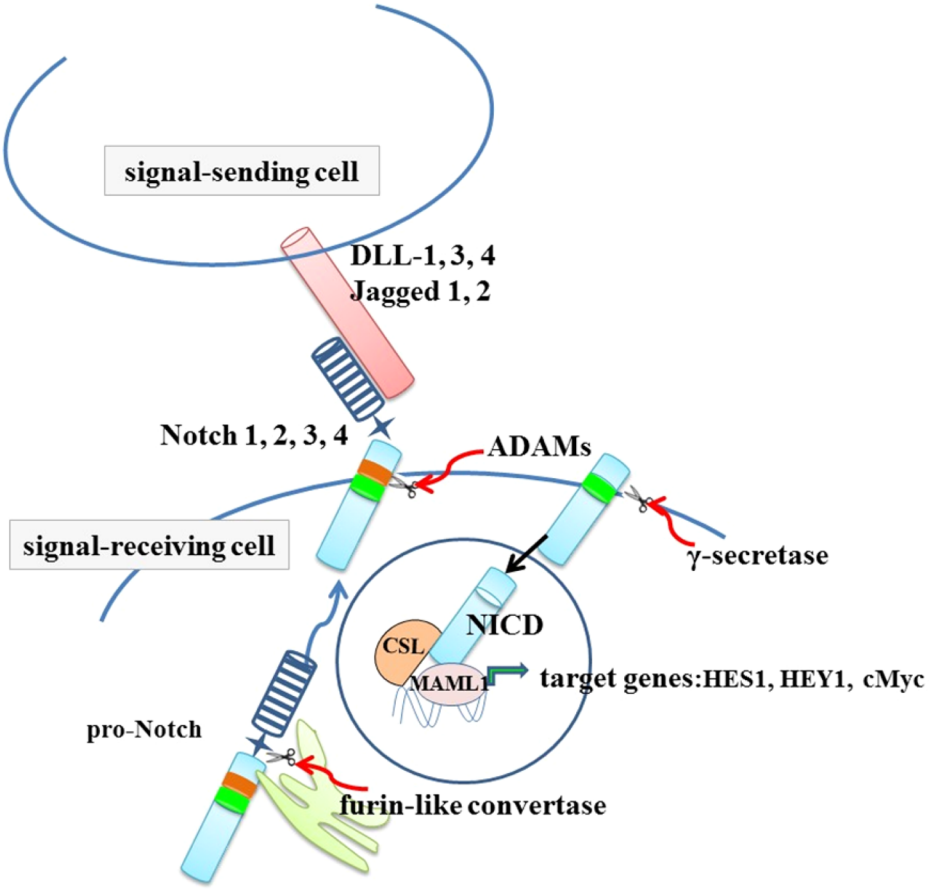

Figure 1. Notch receptors and ligands: canonical activation and signal transduction pathways. There are four Notch receptors (Notch 1−4) and five ligands (Jagged 1 and 2 and DLL-1, 3, and 4) in mammals. In Notch-expressing cells, Notch receptors need intracellular processing. First intramolecular proteolytic cleavage of the newly synthesized precursor receptors by furin-like convertases occurs within the Golgi apparatus, generating heterodimeric Notch receptors. Notch signaling is activated when ligand-expressing cells and Notch-expressing cells interact. The Notch heterodimer interaction with Notch ligands induces two subsequent proteolytic cleavages of the Notch receptor. One by ADAMs, which liberates the extracellular domain, followed by γ-secretase within the transmembrane domain, which results in the release of the intracellular Notch fragment, NICD. NICD translocates to the nucleus and binds to a nuclear protein complex, thereby displacing the corepressor. This is accompanied by the recruitment of coactivator proteins, including MAML1, which activates the transcription of Notch target genes.

its own cell (cis-inhibition).[5,6] Notch activation requires three sequential proteolytic steps. The first proteolytic cleavage (the S1 cleavage) occurs intracellularly in the Notch ectodomain by a furin-like convertase leading to the formation of an intramolecular heterodimeric cell-surface receptor that is protease-resistant in the absence of Notch ligands. Then, ligand binding to the extracellular domain of the Notch receptor initiates two sequential proteolytic cleavage events. The first cleavage of these subsequent steps (the S2 cleavage) is catalyzed by the ADAM family metalloproteinases; the second (the S3 cleavage) is mediated by the transmembrane γ-secretase complex, resulting in the cytoplasmic release of Notch intracellular domain (NICD),[7−9] which translocates into the nucleus where it interacts with members of the CSL family of DNA-binding transcription factors, such as the recombining binding protein suppressor of hairless (RBP)-Jκ (also known as C-promoter binding factor-1, CBF1)[10] as well as its coactivator proteins from the mastermind-like (MAML) family and histone acyltransferases.[11,12] This leads to the formation of the short-lived NICD-CSL-MAML transcriptional activating complex that promotes the transcription of Notch-dependent target genes.[13,14] In the absence of Notch signaling, RBP-Jκ acts as a repressor of transcription by binding to DNA in a sequence-specific manner.

Noncanonical Notch Signaling. The concept of non-canonical Notch signaling followed some in vitro studies where increased Notch 1 expression inhibited muscle cell differentiation but without upregulating the expression of known CSL-dependent target genes, such as RBP-Jκ or hairy and enhancer of split 1 (HES-1).[15,16] Although canonical Notch ligands are predominantly responsible for Notch signaling in physiological and some pathological conditions, structurally unrelated noncanonical ligands have been identified and shown to be associated with cancers and inflammatory diseases.[2,16−20]

1.2. Regulation of Notch Signaling. Notch signaling regulates the expression of target genes in a context- and cell-dependent manner. The dysregulation of Notch activation more commonly leads to oncogenesis; however, in some conditions, Notch activation has tumor suppressive functions. Several studies have been conducted, including genome-wide chromatin immunoprecipitation assays and sequencing to identify genes regulated by Notch.[21,22] Well-studied transcriptional targets of Notch-RBP-Jk include *Hes-1*, the Notch-related ankyrin repeat protein (*Nrarp*), *c-Myc*, and *Deltex*.[23]

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The existence of various specific Notch receptors and ligands allows fine-tuning of the amplitude and duration of Notch activity to generate context- and tissue-specific signals. Downstream signaling is also controlled by post-transcriptional modifications, including glycosylation, phosphorylation, and ubiquitination. Notch is phosphorylated by the following three kinases: glycogen synthase kinase (GSK)-3β,[24] cyclin-dependent kinase (CDK)8,[25] and atypical protein kinase C (aPKC).[26] In physiological conditions, Notch activation is transient because the NICD is phosphorylated after nuclear translocation, allowing interaction with a specific ubiquitin ligase (F-box and WD repeat domain-containing 7, FBW7) and ubiquitin-proteasome-mediated degradation.[27,28] Thus, ubiquitin ligases are thought to function as tumor suppressors. Indeed, mutations in FBW7 and prolonged half-life of NICD were found in T-ALL patients resistant to γ-secretase inhibitors (GSIs)[29,30] as well as in other solid tumors, such as ovarian, breast, and colorectal cancers.[28,31] Furthermore, Notch signaling activation can also be regulated by ubiquitination of either the Notch receptor directly or its ligand expressed at the surface of the neighboring cell, thus promoting their endocytosis.[32,33]

2. NOTCH SIGNALING AND ONCOGENESIS

Notch signaling plays a crucial role in the regulation of cell proliferation, differentiation, and apoptosis. Notch receptors and their ligands are overexpressed in many human cancers.[34] For example, the Notch signaling pathway is involved in normal breast development, and the cell fate of normal breast stem cells, but also in the survival, proliferation, and progression of breast cancer and breast cancer stem cells. Therefore, very complex and subtle pathways of control regulating Notch functions in cancer are required to design and develop therapeutics.

2.1. Cell Cycle and Proliferation. The cell cycle comprises a series of coordinated events resulting in cell division. The cell cycle machinery regulates cell proliferation, and cancer is known to be a disease of altered cell proliferation. It was demonstrated that cyclin D1 (CCND1) transcription and CDK2 activity were induced by Notch in a CSL-dependent manner.[35] Genomic approaches demonstrated that hormone receptor-negative breast cancer cell lines express increased levels of Jagged 1 and identified CCND1 among Jagged 1-regulated genes.[36] It was shown that Jagged 1 downregulation decreases direct binding of Notch to the CCND1 promoter, thus reducing CCND1 expression and inhibiting cell cycle progression through the CCND1-dependent G1/S checkpoint. The proto-oncogene c-Myc, known to drive increased cell proliferation and to downregulate apoptosis, was identified as a direct downstream target of Notch 1.[23]

2.2. Apoptosis. Cell death is decreased in cancer. Notch regulates cell death through extensive networks and signaling pathways of the cell cycle, cell growth, and cell survival, including p53, nuclear factor-kappa B (NF-κB), and phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR).[37] Activated Notch signaling increases the expression of antiapoptotic genes like B-cell lymphoma-2 (*Bcl-2*) and the inhibitor of apoptosis gene family member, *survivin*.[38,39] It was reported that Notch 1 function is required for tumor initiation via suppression of apoptosis through the regulation of p53 stability.[40] In human breast cancer cell lines, increased RBP-J_K-dependent Notch signaling was sufficient to transform normal breast epithelial cells by a mechanism most likely involving suppression of apoptosis. Aberrant activation of Notch 1 and Notch 4 signaling led to the accumulation of NICD, whereas attenuation of this signaling reverted the transformed phenotype of human breast cancer cell lines, suggesting that inhibition of Notch signaling may be a therapeutic strategy for this disease.[41] Downregulation of Notch 3 expression using small hairpin RNA (shRNA) correlated with significant apoptosis and inhibition of proliferation of T-ALL cells.[42] Recently, Notch 1 signaling has also been shown to control cell proliferation, cell death, and differentiation in lung carcinoma.[43] Interestingly, although Notch signaling has generally been associated with tumor growth, recent findings suggest that the effect may be context-dependent and determined by the type of Notch receptor−ligand interaction. Indeed, Notch 2 activation was shown to result in potent inhibitory signals and to induce apoptosis in human breast cancer xenografts.[44]

2.3. Angiogenesis. The formation of new blood vessels is an important step for tumor growth and metastasis. Vascular endothelial growth factor (VEGF), one of the best-characterized inducers and key regulators of tumor angiogenesis, is induced by hypoxia-inducible signals in the tumor environment. In hypoxic conditions, Notch activity is also potentiated by the accumulation of hypoxia-inducible factors (HIF)-1α and -2α, which synergize with Notch coactivator MAML1 to promote epithelial-to-mesenchymal transdifferentiation (EMT) and thereby metastasis. In line with these mechanisms, inhibition of either Notch or HIF results in reduced invasion and metastatic potential of tumor cells.[45,46] Importantly, Notch signaling components are known to be expressed in endothelial cells and play an important role during vascular development.[47−49] Notch ligands (in particular DLL) have been shown to be highly expressed in tumor vasculature and to positively correlate with VEGF and CD34 expression levels.[50] Similarly, the level of Jagged 1 expression was shown to correlate with tumor blood vessel content and to associate with the progression of head and neck squamous cell carcinomas.

2.4. Epithelial-to-Mesenchymal Transdifferentiation (EMT). EMT is a fundamental process during embryonic development whereby epithelial cells lose polarity and intercellular adhesion, adopt a mesenchymal phenotype, and therefore acquire migratory properties. EMT is pathologic however in adult tissues, leading to fibrosis, and is often activated during cancer, contributing to tumor progression and metastasis.[51,52] In epithelial and endothelial cells, Jagged 1/Notch activation results in morphologic and functional changes consistent with mesenchymal transformation.[53] These changes include attenuation of many endothelial markers (endothelial cadherin (E-cadherin), endothelial nitric oxide synthase (eNOS), and platelet-endothelial cell adhesion molecule-1 (PECAM-1)), upregulation of several mesenchymal markers (alpha smooth muscle actin (α-SMA), fibronectin, and platelet-derived growth factor (PDGF)-receptor)), increased migration, resistance to apoptosis, and invasiveness.[54,55] Moreover, the loss of the epithelial phenotype through EMT can promote the acquisition of a stem-like phenotype and drug resistance.[56,57] By promoting EMT, Notch signaling also regulates the formation of cancer stem cells (cells within a tumor that possess the capacity to self-renew and differentiate into the heterogeneous lineages of cancer cells that comprise the whole tumor), which were shown to be associated with tumor progression, metastasis, and recurrence.[58,59]

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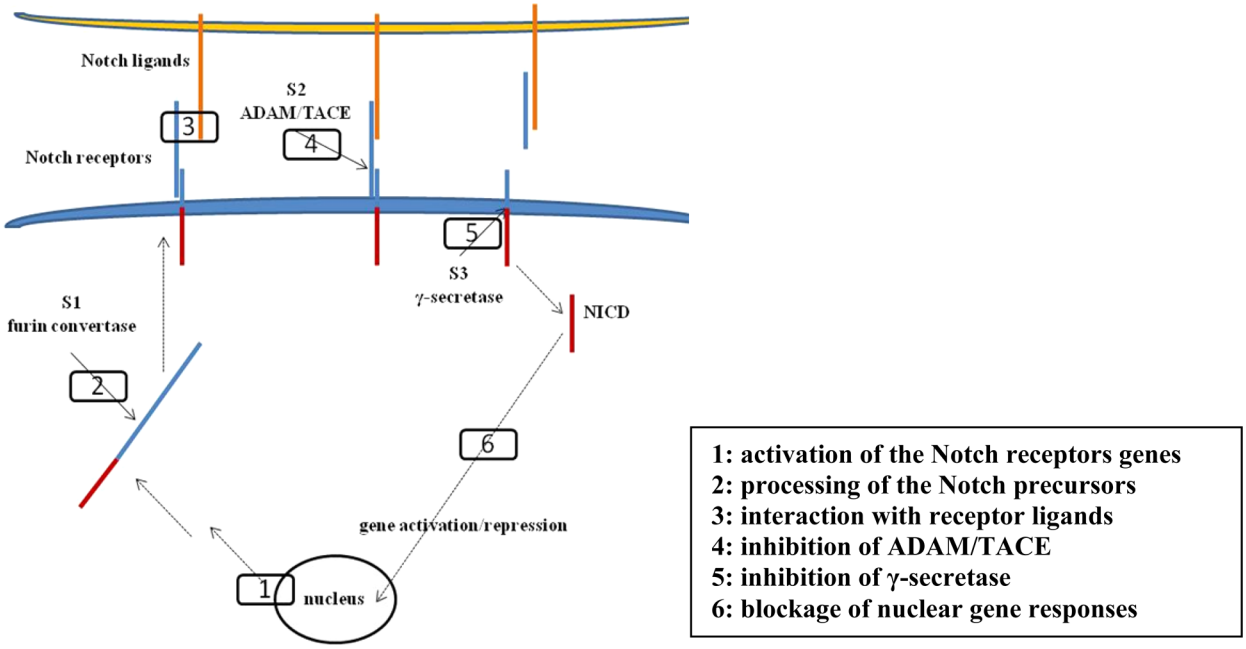

Figure 2. Biosynthesis of the Notch receptors and potential control of their activation. Schematic representation of the Notch activation pathways in cells and the potential points of control for the modulation of Notch-dependent functions.

3. NOTCH AS A TUMOR SUPPRESSOR

Although Notch has been mostly implicated in oncogenic pathways, recent data suggest tumor suppressive functions in specific organs, such as the liver, hematopoietic stem cells, and the skin.[13,60] In a mouse model of hepatocarcinoma (HCC),[61] inhibition of Notch signaling using the GSI DAPT[62] (**1**, Table 1) resulted in accelerated cancer development, and enforced expression of NICD in HCC cells promoted cell cycle arrest in G2 phase and enhanced apoptosis. Investigating cohorts of HCC patients, the same authors observed better survival in patients with significantly higher expression of *Notch1* and its target gene *Hes1*. In line with these data, Notch inhibition was reported to promote angiogenesis and growth of hepatic metastases.[63] Whole-exome sequencing and gene copy number analyses performed in head and neck squamous cell carcinomas identified mutations in *Notch1* predicted to truncate the gene product leading to inactivation.[64] Notch ligands and receptors are also expressed in the skin where Notch signaling triggers pathways leading to keratinocyte growth arrest and differentiation as suggested by data showing that keratinocyte-specific deletion of the *Notch1* gene leads to marked epidermal hyperplasia.[65]

4. NOTCH SIGNALING IN THE KIDNEY

Recent studies of renal diseases have yielded several candidate pathways for designing cell-targeted therapeutics, which include the Notch pathway.[66−71] Within the kidney, injury to glomerular or tubular cells is the initiating cause of many acute and chronic diseases, leading to progressive dysfunction and end-stage renal disease. The glomerulus is the main filtration barrier that determines global kidney function. Inflammatory and noninflammatory stress affects the glomerulus and leads to alterations in its structure, permeability, and function, resulting in chronic kidney disease. Injury to the tubulo-interstitial tissue is a major cause of acute kidney disease, particularly in weakened hospitalized patients. Genetic studies performed in mice with conditional expression of the active Notch 1 protein showed massive glomerulosclerosis, leading ultimately to renal failure and death of the animals. Genetic deletion of Notch transcriptional binding partners or treatment with GSIs, preventing Notch activation and translocation to the nucleus, protected the animals from nephrotic syndrome. Thus, targeted pharmacologic inhibition of the Notch signaling pathway may prevent kidney damage in a variety of diseases.

5. NOTCH SIGNALING AND FIBROSIS

Fibrosis is a nonspecific terminal pathway following local inflammation and scarring. It is the hallmark of many chronic inflammatory diseases, as well as a predictor of progressive organ dysfunction.[72,73] The pathogenesis of fibrosis involves an initial and probably repetitive tissue injury/inflammation that leads to abnormal tissue repair involving locally recruited inflammatory cells (in particular, macrophages) and resident mesenchymal cells, such as fibroblasts and myofibroblasts, resulting in thickening of the interstitial tissue and functional impairment. The increased number of (myo)fibroblasts could originate from either excessive proliferation and acquired resistance to physiological apoptosis or be the consequence of local EMT,[74−76] all processes that can be regulated by Notch signaling. For example, in kidney diseases and after kidney transplantation, tubulo-interstitial fibrosis (TIF) is considered as the final common pathway (regardless of the primary lesion) leading to kidney dysfunction. The TIF severity score is highly prognostic of organ survival.[77,78] In experimental models, tubular epithelial cell-specific expression of active Notch 1 caused rapid development of TIF mainly through the process of EMT, whereas specific genetic deletion or pharmacologic inhibition using the GSI DBZ/deshydroxyLY-411575[66,79] (**2**, Table 2) ameliorated TIF.[66] Transforming growth factor (TGF)-β is a growth factor secreted by immune cells recruited to inflamed/injured tissues contributing to tissue repair. In cultured human proximal tubular epithelial (HK-2) cells, TGF-β1 was shown to induce the expression of fibronectin (a stimulant and chemotactic agent for fibroblasts) as well as other

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factors involved in fibrosis and EMT, including the Notch ligand Jagged 1.[80] Overall, current data indicate that epithelial Notch signaling regulates interstitial fibrosis, and its blockade could be a therapeutic strategy to prevent end organ diseases.

6. NOTCH SIGNALING AND REPRODUCTIVE BIOLOGY

Members of the Notch signaling pathway are expressed in mammalian ovaries and are important regulators of developmental pathways. Primary ovarian follicles cultured in vitro and treated with the GSIs **1** or L-658,458[81,82] (**3**, Table 2) stopped their Notch-dependent development. Similarly, **1** or **3** inhibited proliferation of cultured primary granulosa cells. The Notch ligand DLL 4 is involved in normal luteal vasculature, and Notch signaling plays an important role in regulating progesterone secretion in murine luteal cells. Murine luteal cells treated with **1** or **3** demonstrated decreased chorionic gonadotropin (hCG)-stimulated progesterone secretion, whereas overexpression of the intracellular domain of Notch 3 increased progesterone secretion.[83,84]

7. THERAPEUTIC INTERVENTIONS TARGETING NOTCH

Understanding of the Notch signaling pathway has reasonably increased, and its role in diverse pathological conditions has drawn attention for pharmacological interventions.[85] Current strategies include disrupting the proteolytic cleavage/processing of Notch or inhibition of Notch−ligand interactions.

Potential Points of Intervention in the Notch Pathway. As stated above, the Notch family of receptors comprises four members, Notch 1−4, with different, and sometimes antagonizing, roles in modulating cell and tissue functions, such as cell fate, proliferation, growth, and differentiation (Figure 2). The four Notch receptors have different patterns of expression depending on the cell type and state of differentiation. Therefore, the exact choice of the therapeutic target is of utmost importance. Consequently, it is necessary to define whether pan-Notch or receptor-selective therapeutics are most appropriate.

Notch receptors are large single-pass type I transmembrane glycoproteins expressed as a heterodimer produced by proteolytic processing from a single chain *O*-glycosylated monomeric precursor (~300 kDa). The four mammalian Notch receptors have different sizes: Notch 1 is the largest and Notch 4 the smallest. All four receptors are comprised of three domains: extracellular, transmembrane, and intracellular. The extracellular N-terminal domains are the ligand binding part of the receptors and are constituted of several (29−36) EGF-like repeats and three disulfide bridges. The extracellular juxtamembrane domain regulates heterodimerization, maintaining the receptors in nonactivated states. The transmembrane domain contains the γ-secretase cleavage site. The intracellular C-terminal domain extends from the inner cell membrane into the cytoplasm and contains several regulatory elements, preventing ligand-independent interactions, and the transcriptional activator domain. Following binding of one of the Notch receptor ligands, a conformational change in the receptor occurs, allowing initiation of the activation of the Notch intracellular pathways and transcriptional program.

The vertebrate Notch ligands are represented by two families of single-pass type I transmembrane-inserted proteins, DLL-1, 3, and 4 and Jagged 1 and 2, with intracellular and extracellular domains. The extracellular domain is the binding domain comprising 6−10 EGF-like repeats, and Jagged ligands bear an additional cysteine-rich domain. The ligands for Notch receptors are expressed by cells different from the cells expressing the receptors. Cell−cell contact allows interaction between Notch receptors and their ligands mediated by the EGF-like repeats of both receptors and ligands. This interaction initiates a conformational change (the "pull") in the Notch receptors, exposing a cleavage site (the S2 site) for ADAM metalloproteinases, leaving approximately 12 amino acids protruding on the extracellular membrane. The remaining transmembrane protein is then subjected to further proteolytic processing mediated by the membrane-inserted aspartyl protease γ-secretase at amino acid Val1744. This proteolytic step results in the release of NICD from the membrane into the cytoplasm, where it then translocates into the nucleus. NICD forms a transcriptional activation complex in the nucleus, leading to increased expression of specific genes, including *c-Myc*, *p21*, *CCND1* (cell cycle progression), *Bcl-2* (inhibition of apotosis), as well as genes of the *Hes* and *Hey* families (mediating cell fate). Then, the NICD is phosphorylated in the nucleus and marked for degradation by the proteasome.[85]

Below, we will review various attempts to modulate the Notch signaling pathway for therapeutic intervention in human diseases, in particular, drugs that have been evaluated in clinical trials.

Where Is It Possible to Act to Modulate the Functions of the Notch Signaling Pathway? *a. Regulating Activation of the Genes and Translation of the Notch Receptors and Ligands.* For blocking (or activating) the *Notch* genes, several approaches have been attempted using gene silencing by shRNAs, siRNAs, miRNAs, or modification of histone chaperones acting as gene silencing. However, to the best of our knowledge, these tools have been used only in in vitro cell or animal experimental models. Although this is very useful to define the role of Notch in physiological and pathological situations or to validate experimental tools, no attempts have been made to exploit these tools in the clinic. Notch signaling can also be modulated downstream of Notch receptor activation by other pathways, such as the PI3/Akt, GSK3, or EGFR pathways, which may also be used to indirectly modify the Notch signaling pathway.

b. Inhibiting the Processing of the Precursors of the Notch Receptors by Furin-like Proteases, Glycosylation Pathways, and Exit from the ER/Golgi. Glycosylation pathways and processing of precursor proteins by furin-like convertases are fundamental pathways in the homeostasis of all tissues and cells and in the processing of many secreted proteins. Thus, very specific and selective tools are required to target Notch glycosylation or processing by furins in defined diseased cells. To the best of our knowledge, the role of these pathways has only been determined in in vitro cellular or animal experimental models. No attempts at targeting Notch glycosylation have been made in the clinic. A few furin inhibitors have been developed (see below) and evaluated in animal models not involving Notch processing. These compounds may also be of interest in the control of the Notch signaling pathway, if they can be rendered specific and selective for defined cells.

Furins (PACE, paired basic amino acid cleaving enzyme, EC 3.4.31.75) are a family of calcium-dependent serine endoproteases biosynthesized as inactive proenzymes that need self-mediated intrachain cleavage to become enzymatically active. Furins belong to the subtilisin-like proprotein convertase family. The members of this family process latent precursor

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proteins into their biologically active products,[86,87] including the TGF-β1 precursor, pro-β-secretase, membrane type-1 matrix metalloproteinase, the HIV envelope polyprotein precursor gp160 to gp120 and gp41, and Notch precursors. Furins have also been involved in tumor progression. Furins are mainly located within the Golgi/trans-Golgi secretory pathway where they cleave other proteins downstream of a dibasic amino acid target sequence (Arg/Lys-Arg) into their mature/active forms. Inhibitors of furins have been explored as therapeutic agents for treating anthrax infection. Soluble furins can be inhibited by EGTA, α1-antitrypsin, and polyarginine compounds as well as by a few synthetic molecules.

The catalytic mechanism of enzymes of the serine protease family involves a *catalytic triad* located in the active site of the enzyme. The triad is a coordinated structure consisting of three essential amino acids: one histidine, one serine, and one aspartic acid. In the catalytic mechanism, several intermediates are generated, including a covalent acyl-enzyme intermediate. The released pro-fragments frequently act as inhibitors of the enzyme. Furin inhibitors have been developed, and they include poly-arginine peptides, α1-antitrypsin, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (**4**, furin inhibitor I), and CCG-8294 (**5**) (Figure 3).[88,89] Furin inhibitor I is a selective, irreversible,

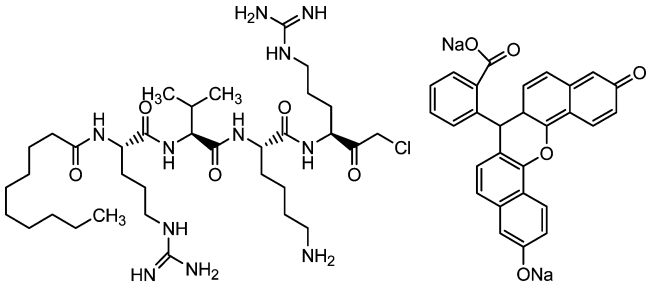

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Figure 3. Examples of Furin Inhibitors.

and cell-permeable competitive inhibitor of proprotein convertases, including furin/SPC1 (K_i = ~1 nM), SPC2/PC2 (K_i = 0.36 nM), SPC3/PC1/PC3 (K_i = 2.0 nM), SPC4/PACE4 (K_i = 3.6 nM), SPC6/PC5/PC6, and SPC7/LPC/PC7/PC8 (K_i = 0.12 nM).

Very recently, in a furin-unrelated approach for controlling the processing of protein precursors in intracellular organelles, FLI-06[90] (**6**, Table 4) has been shown to be an inhibitor of Notch signaling (IC_{50} = 2.3 μM), which also reduces amyloid-β (Aβ) secretion. Compound **6** acts upstream of α-secretase and β-secretase cleavage, inhibiting endoplasmic reticulum (ER) export and thus disrupting intracellular trafficking and processing of Notch and general secretion at a step before exiting the ER. Therefore, **6** is the first small molecule acting at such an early stage in the secretory traffic. This inhibition is accompanied by a tubule-to-sheet morphological transition of the ER. Compound **6** does not act on the cytoskeleton but causes a complete disruption of the Golgi in a manner different from that of Brefeldin A or Golgicide A. These data highlight the power of phenotypic screening for investigating central cellular signaling pathways. Obviously such an approach would need further validation to evaluate its clinical interest and potential and again very tight mechanisms of specificity and selectivity of delivery to the target cells.

c. Controlling the Interaction between Notch Receptors and Their Ligands. Notch signaling can also be blocked more specifically by using monoclonal antibodies (mAbs) against individual Notch ligands or receptors overexpressed in diseases.[91−94] Inhibition of Notch signaling using a mAb targeting DLL-4 resulted in reduced tumor growth mainly by controlling angiogenesis, as DLL-4 expression in endothelial cells is dynamically regulated by VEGF. These findings also indicated that DLL4-mediated Notch signaling is predominantly restricted to the vascular compartment and that targeting DLL-4 may have limited adverse impact on other cells, including intestinal cell differentiation. The selective blockade of Notch 1 activation using a mAb against the human Notch 1 ligand-binding domain (EGF-repeats 11−15) decreased breast cancer stem-like cell proliferation and induced apoptosis.[94,95]

The binding of a ligand to a Notch receptor induces a conformational change of the receptor, which is the result of a "pull" force on the ligand to be internalized by endocytosis, exposing a site on the receptor for proteolysis by ADAMs. Conversly, the agonist 256A-13 (structure not disclosed) can induce the proteolytic cleavage of Notch 3[96] by mimicking the effects of ligand binding to Notch. Antagonists able to bind to the Notch receptors without inducing the "pull" and the subsequent proteolytic step have also been developed.

d. Inhibiting Notch Cleavage By ADAMs. ADAMs, and metalloproteinases in general, have been the target of several campaigns of drug development and clinical evaluation. Several families of enzyme-selective inhibitors have been designed and may present some interest in the context of the control of Notch activation. However, to the best of our knowledge, none of them has reached clinical trials for Notch blockade, although some of them have been evaluated in preclinical Notch experimental models.

Members of the ADAM metalloproteinases are cell surface proteins with a unique structure possessing both potential adhesion and protease domains. Sheddases, a generic name for the ADAM metalloproteinases, function primarily to cleave membrane proteins at the cell surface, releasing soluble ectodomains of their protein substrates. Although a single sheddase may "shed" a variety of substrates, multiple sheddases can cleave the same substrate, resulting in different consequences. Although the exact enzymatic mechanism of ADAMs has not been thoroughly investigated, their active sites are comparable to those of well-studied zinc proteases, such as carboxypeptidase A and thermolysin. Therefore, it is proposed that ADAMs utilize similar mechanisms as these enzymes. In zinc proteases, the key catalytic elements consist of a Zn^{2+} ion, two histidines, and a glutamic acid. The water molecule is hydrogen-bonded to another glutamic acid in a relay system with either a serine or a histidine of the enzyme. The proposed mechanism begins with deprotonation of the water molecule by the glutamic acid. The resulting hydroxide initiates a nucleophilic attack on a carbonyl carbon of the peptide backbone, producing a tetrahedral intermediate, leading to the first transition state and the formation of the tetrahedral intermediate. Following the formation of the transition state, the leaving group is protonated, and the peptide bond is cleaved.

ADAM metalloproteinase domain 17 (ADAM17/TACE, tumor necrosis factor-α-converting enzyme, EC 3.4.24.86) is a 70 kDa protein involved in the shedding of tumor necrosis factor alpha (TNF-α) at the surface of cells and in the

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membrane of the trans-Golgi network. ADAM17 releases other membrane-anchored cytokines, cell adhesion molecules, receptors, ligands, and enzymes or regulates the MAP kinase signaling pathway by controling the shedding of the EGFR ligand amphiregulin. ADAM17 also hydrolyzes the full-length amyloid precursor protein (APP) to the soluble N-terminal fragment. Functional ADAM17 is ubiquitously expressed in the human colon and is overexpressed in digestive tract diseases.[97−99] The ADAM17 inhibitor TAPI-1[100] (7, TNF-α protease inhibitor 1, Figure 4) is an inhibitor of matrix metalloproteinases (MMPs), including ADAM17/TACE, which blocks the shedding of cell surface proteins with an IC_{50} = 8.1 μM for TNF-α.

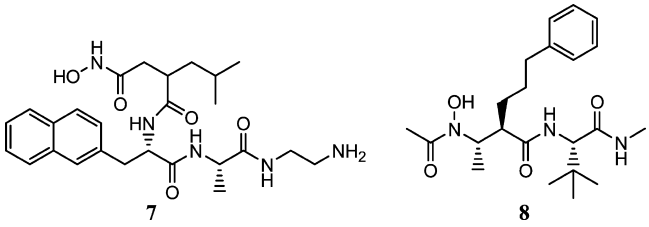

Figure 4. Examples of ADAM inhibitors.

ADAM metalloproteinase domain 10 (ADAM10, CDw156 or CD156c, EC 3.4.24.81) has a broad specificity for peptide hydrolysis, including TNF-α, ephrin, APP, CD44, HER2, and Notch.[101,102] The ADAM10 inhibitor GI254023X[103] (**8**, Figure 4) is potent and selective as it displays over 100-fold higher potency for ADAM10 compared to ADAM17 (IC_{50} = 5.3 nM for ADAM10 versus 541 nM for ADAM17/TACE) and blocks the constitutive shedding of cell surface proteins in cells. In combination with low doses of the anti-HER2 herceptin, selective ADAM10 inhibitors decrease proliferation of HER2-overexpressing tumor cells.

e. Inhibiting S3 Cleavage by γ-Secretase. The use of GSIs has been the most explored way of controlling Notch activation. The search for GSIs started with the aim of inhibiting the processing of APP by γ-secretase in Alzheimer's disease (AD).[104−106] AD is characterized by the loss of neuronal functions, the presence of aggregates of extracellular plaques of Aβ (Aβ_{1-40} and Aβ_{1-42}), and intercellular neurofibrillary tangles of phosphorylated tau protein, which have been postulated to be responsible for the development of AD. The Aβ aggregates are produced by the sequential action of two aspartyl proteases, β-secretase (BACE), and γ-secretase on APP. The γ-secretase cell membrane-inserted protease complex has the capacity to cleave the APP transmembrane domain inside the cell membrane. BACE and γ-secretase are found throughout the secretory pathway and at the cell surface. Therefore, these two aspartyl proteases are obvious targets to develop therapeutic interventions for AD, and inhibitors for these two proteases have been developed and clinically evaluated.[107,108] In the context of AD,[105,106] preclinical and clinical evaluations of GSIs revealed unwanted side-effects attributed to the inhibition of Notch processing.

The enzyme γ-secretase is a large protease complex composed of four transmembrane proteins: three support proteins, Pen-2 (presenilin enhancer protein-2), Aph-1 (anterior pharynx-defective-1, of which 3 isoforms exist), and Nct (nycastrin), and the catalytic subunit presenilin (PS, EC 3.4.23.-), of which two isoforms exist, PS-1 and PS-2. Thus, several γ-secretase complexes may be assembled with slightly different substrate specificities, but all four proteins are obligatory for the enzymatic activity of γ-secretase. In humans, the *PSEN-1* gene is located on chromosome 14 and the *PSEN2* gene on chromosome 1. PS-1 and PS-2 share high sequence homology and have nine transmembrane domains with a cytosolic amino-terminus and a luminal carboxy-terminus. PS-1 and PS-2 are the catalytic part of the γ-secretase complex with the two catalytic aspartic acids being located in the membrane lipid bilayer in transmembrane domains 6 and 7, which are close to each other in a water-containing cavity.[109,110] The presence of these two close aspartic acid residues and the water molecule is an important feature of the complex because the catalytic mechanism of aspartyl proteases (which includes renin, HIV protease, cathepsin D, and BACE) requires the presence of a water molecule activated by two carboxylates. This activated water molecule directly attacks the peptide bond, producing a transition-state intermediate without the formation of a covalent intermediate. Substrates of γ-secretase first bind to Nct; then, the catalytic subunits of the γ-secretase complex perform an intramembrane hydrolysis.[111] However, very recently and in contrast to previous studies, it was shown that the substrate transmembrane domain drives its interaction with γ-secretase. The γ-secretase component Nct sterically blocks substrates with large ectodomains from interacting with γ-secretase, providing the mechanism by which γ-secretase selectively recruits short ectodomain substrates. γ-Secretase-substrate binding is driven by an apparent tight-binding interaction derived from the substrate transmembrane domain.[112] γ-Secretase cleaves single-pass transmembrane proteins and has over 100 identified and postulated substrates, which includes Notch. The γ-secretase complex activates Notch by hydrolyzing a peptide bond of the Notch protein at an intramembrane site, allowing the cleaved NICD to migrate to the nucleus where it activates responsive genes.[113] The intramembrane activity of the γ-secretase has also been involved in the release from the membrane of other biologically relevant membrane proteins involved in normal and pathological processes, including insulin-like growth factor or sorting receptors.[114,115] Therefore, for selective therapy, it is mandatory to achieve only localized inhibition of this activity, thus protecting the other functions of this enzyme complex and the normal physiological functions of the Notch pathway in particular.

More than a thousand[34,109] different types of GSIs have been designed, synthesized, and evaluated, including competitive active site binders, non/uncompetitive substrate docking site binders, and alternative site binders. These include various peptides isosteres, benzazepines, sulfonamides, and others. Many of them are orally active and have low nanomolar IC_{50} values, and several of them have been evaluated in clinical trials. They can be either transition-state analogues (targeting the active site of the enzyme like the substrates) or non-transition-state analogues (targeting the active site of the enzyme or binding sites different from the enzyme active site), benzazepines, sulfone analogues, or tetralin imidazoles (Tables 1 and 2).

f. Blocking the Transcriptional Response Induced by the NICD. For blocking (or activating) the transcriptional functions of Notch, several approaches can be attempted using gene silencing or decoy proteins of the components of the transcriptional complex. However, to the best of our knowledge, these tools have been used only in in vitro cellular or

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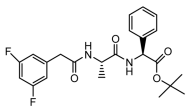
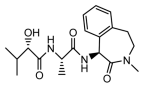
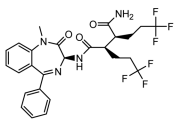
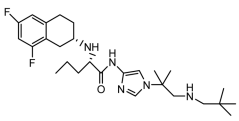
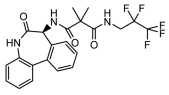
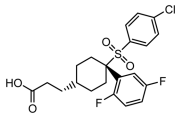
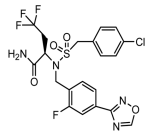
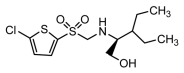
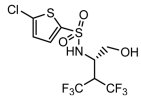
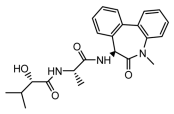
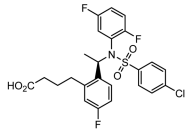
Table 1. γ-Secretase Inhibitors of Clinical Interest for Therapeutic Blockade of the Notch Pathway in Cancer

code/name	chemical structure	company	clinical trials	[references]
1 DAPT		Eli Lilly	GSI most widely used experimentally	62,105
9 LY-450139 semagacestat		Eli Lilly	phase I (development stopped in 2010 in phase III for AD)	123,124
10 BMS-906024		Bristol-Myers Squibb	phase II	125
11 PF-030840		Pfizer	phase II	126-128
12 RO4929097		Hoffmann-LaRoche	phase I/II	119,129
13 MK-0752		Merck	phase I/II	108,130,131
14 BMS-708163 avagacestat		Bristol-Myers Squibb	phase I (development stopped in 2012 in phase II for AD)	132-134
15 GSI 136		Wyeth / Pfizer	phase I	135,136
16 GSI-953 begacestat		Wyeth	phase I (tested in normal volunteers)	137
17 LY-900009		Eli Lilly	phase I (phase 1 stopped for adverse events)	138
18 BMS-299897		Bristol-Myers Squibb	phase I/II	139,140

animal experimental models. No attempts have yet been made to test these in the clinic.

8. PRECLINICAL AND CLINICAL TRIALS AIMED AT CONTROLLING THE NOTCH SIGNALING PATHWAY IN DISEASES

Current approaches in preclinical and clinical trials[116] include inhibiting the proteolytic processing of the Notch receptor and/or interfering with the ligand−receptor interaction. Auxillary proteins, such as glycosylases, kinases, or ligases, involved in the Notch pathway also offer new possibilities of control. In addition, some biotechnological tools have been developed; for example, recent patents related to Notch signaling have been published, which include transfection of cells with the NICD for antiviral purposes (Academy of Military Medical Sciences, CN103386137) or a Notch 3 fusion protein as a decoy for cancer (Columbia University, SG193873).[117]

GSI-Based Therapeutics. As the Notch ligand−receptor binding interaction triggers intramembranous cleavage of the Notch receptors by the γ-secretase complex, targeting this complex using pharmacological inhibitors is the most studied approach to effectively inhibit the production of NICD and the transcription of Notch target genes.[106] Following encouraging results obtained using human cancer cell lines in vitro together with xenograft experimental models,[118−121] ongoing clinical trials are now investigating the safety and potential efficacy of GSIs, alone or in combination with other drugs, in various types and stages of cancer or inflammation. During the development and evaluation of GSIs for AD, undesirable side effects were observed, which were attributed to the poor selectivity of these GSIs and their interference with the processing of AD-unrelated protein substrates by γ-secretase, in particular, components of the Notch signaling pathway. The off-target side effects observed for GSIs included gastrointestinal toxicity,[122] cognitive worsening, and an increased risk for some cancers. These observations resulted in the termination of clinical trials of, for example, semagasestat/LY-450139[123,124] (**9**, Table 1) in phase III. Therefore, in some situations, GSIs are required to possess an increased selectivity for Notch as compared to the myriad of other γ-secretase substrates.

In cancer, and in particular cancers of immune cells such as acute myeloid leukemia (Notch silenced) or T-ALL (Notch constitutively activated), the Notch signaling pathway has been shown to have both pro-tumoral (oncogenic) and tumor suppressive functions. These effects depend on the cell type, the stage and grade of the tumor, and the cellular context, including the role of the stromal environment of the tumor (angiogenesis, immune response, fibrosis) as well as the presence of mutations in the different components of Notch and its associated cellular pathways, such as apoptotic and proliferative responses. Therefore, under some situations, the Notch pathway needs to be therapeutically blocked, whereas in others it should either be induced or activated. The choice of which of the Notch receptors and ligands must be modulated also needs to be defined, and presently, it is not always clear

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what factors dictate this choice. Several GSIs have been evaluated in human clinical trials (Table 1).

These GSIs are all able to inhibit γ-secretase with low nanomolar efficacy. However, for evaluating their efficacy, very different biological models have been used, including cell-based, cell-free, or animal models; no standardized protocols have been used, and no reference molecule for comparison has been included in the assays. For some of them, only APP processing has been determined. Therefore, directly comparing their potency toward Notch inhibition is at best problematic. Thus, the IC_{50} values provided below, when available, must be used with caution.

Compound **1**[62,105] is a non-transition-state analogue GSI and is the compound most widely evaluated in experimental models. It causes a reduction in Aβ levels in human primary neuronal cell cultures with IC_{50} values = 115−200 nM. Compound **1** blocks Notch signaling in a cell-based assay measuring the activation of the Notch pathway reporter gene with an IC_{50} = 500 nM. As an inhibitor of Notch, **1** has also been used in the study of autoimmune and lymphoproliferative diseases. Compound **9**[123,124] is an orally available GSI (Aβ: IC_{50} = 10.9−12.1 nM; Notch: IC_{50} = 14.1 nM) that was developed as a candidate therapeutic drug against AD (IDENTITY trial), which failed in phase III trials. BMS-906024 (**10**, Table 1)[125] is a new pan-Notch GSI disclosed by Bristol-Myers Squibb at the 2013 Spring ACS meeting. The structure is one of a set patented in 2012 and is currently being studied in Phase I clinical trials both alone and in combination with other drugs to treat breast, lung, and colon cancers and leukemia. PF-03084014 (**11**, Table 1)[126−128] is a non-transition-state GSI (Aβ: IC_{50} = 1.2 nM, cell-based assay; IC_{50} = 6.2 nM, cell-free assay) with potential antitumor activity, inhibiting tumor cell proliferation and survival, inducing apoptosis of tumor cells that overexpress Notch and in xenografts models, reducing endogenous NICD levels, and downregulating the Notch target genes *Hes-1* and *c-Myc* in T-ALL cell lines via cell cycle arrest. Broad antitumor efficacy at well-tolerated dose levels was observed in six Notch-dependent animal models. Further studies of **11**-induced gastrointestinal toxicity identified an intermittent dosing schedule that limited body weight loss while maintaining antitumor efficacy. Glucocorticoid administration abrogated **11**-induced gastrointestinal toxicity without compromising the protective effect. Collectively, current results show that inhibition of Notch signaling by **11** while minimizing gastrointestinal toxicity[128] represents a promising approach for the development of therapies in Notch receptor-dependent cancers. This compound is being investigated in phase I clinical trials for the treatment of T-ALL as well as for advanced solid tumors. RO4929097 (**12**, Table 1)[119,129] is a GSI displaying an IC_{50} = 4 nM in a cell-free assay. Compound **12** inhibits cellular processing of Aβ40 and Notch with EC_{50} = 14 and 5 nM, respectively, in a Notch cell-based reporter assay. The potency of **12** in cell-free and cell-based assays displays >100-fold selectivity with respect to 75 other proteins of various types, including receptors, ion channels, and enzymes (CEREP panel). It was shown that **12** blocked Notch processing in human non-small cell lung carcinoma cells and decreased the expression of the Notch transcriptional target genes. However, **12** only modestly inhibited the growth of cells in a dose-dependent manner (1 μM of **12** induced 10−20% cell growth inhibition depending on the cells). Compound **12** increased T-cell activation and shifted cytokines toward a Th_1 profile. In nude mice bearing A549 lung tumor cell xenografts, oral gavage of 3−60 mg/kg of **12** once daily or twice daily for 7, 14, or 21 days resulted in significant tumor growth inhibition (ranging from 66 to 91%) compared with those of vehicle-treated animals. Inhibition of tumor growth remained prolonged and sustained up to 34 days post-treatment. Compound **12** led to reduced expression of genes associated with angiogenesis in A549 xenografts. In contrast, the **12**-resistant H460a xenograft models displayed little change in expression of these genes, underscoring the in vivo antiangiogenesis mechanism of action of **12**. For IL-6 and IL-8 overexpressing tumors, **12** no longer impacted angiogenesis or the infiltration of tumor-associated fibroblasts. MK-0752 (**13**, Table 1)[130,131] is a non-transition-state analogue GSI that reduces Aβ40 with an IC_{50} = 5 nM in human cells. Compound **13** also inhibits Notch cleavage to NICD and its subsequent nuclear translocation in vitro. It is presently in Phase I/II clinical trials for cancer. BMS-708163/avagacestat (**14**, Table 1)[132−134] is a potent, allosteric, orally bioavailable GSI inhibiting Aβ formation with an IC_{50} = 0.3 nM and Notch activation with an IC_{50} = 58 nM. As Notch inhibition caused side effects that forced the termination of the previous clinically evaluated compound **9**, the drug-development program presently aims to achieve a greater selectivity between APP and Notch inhibition. Compound **14** binds directly to the PS-1 N-terminal fragment, and that binding can

Table 2. γ-Secretase Inhibitors Not (Yet) Evaluated in Clinical Trials for Therapeutic Blockade of the Notch Pathway in Human Diseases or Evaluated for Other Diseases

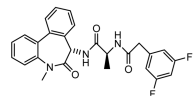
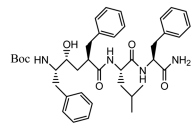
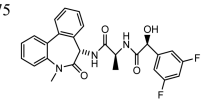
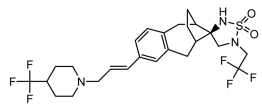
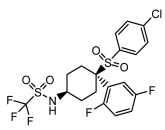
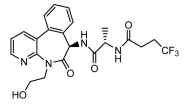
code	structure	IC_{50}	comments	[references]
2 DBZ (deshydroxy-LY-411575) YO-01027		Aβ: 2.6 nM; Notch: 2.9 nM	benzazepine	66,79
3 L-658,458		Aβ:17 nM (cell-free) Aβ:115-200 nM (cell)	transition state analogue	81,82
19 LY-411575		Aβ: 0.08 nM; Notch: 0.39 nM	benzazepine induces apoptosis in Kaposi's sarcoma cells promotes neural and intestinal goblet cell differentiation	142,143
20 MRK-003			non-transition state analogue sulfonamide inhibits Notch3	120,144-146
21 MRK-560		Aβ: 65 nM	sulfonamide selective for Aβ over Notch	147,148
22 LY-3039478		Notch: 0.41 nM	benzazepine	149,150

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Table 3. Ongoing and Completed Clinical Trials of GSIs in Cancer

NCT identifier	phase	therapeutic agent	status	sponsor/collaborators	tumor type
NCT01292655	I	**10**	recruiting	Bristol-Myers Squibb	advanced or metastatic solid tumors
NCT01653470	I	**10** + chemotherapy regimens	recruiting	Bristol-Myers Squibb	advanced or metastatic solid tumors
NCT01363817	I	**10** + dexamethasone	recruiting	Bristol-Myers Squibb	T-ALL or T-cell lymphoblastic lymphoma
NCT02137564	II	**11**	active	AIDS Malignancy Clinical Trials Consortium National Cancer Institute (NCI), The EMMES Corporation	AIDS-related Kaposi sarcoma
NCT01238133	I	**12** + carboplatin + paclitaxel	active	NCI	stage II or III triple-negative breast cancer
NCT01238133		**12** + cediranib maleate	completed	NCI	advanced solid tumors
NCT01154452	I/II	**12** + vismodegib	active	NCI	advanced or metastatic sarcoma
NCT01981551	II	**11**	ongoing	NCI	desmoid tumors/aggressive fibromatosis
NCT01196416	I/II	**12** + cisplatin + vinblastine + temozolomide	ongoing	NCI	recurrent or metastatic melanoma
NCT01088763	I	**12** + dexamethasone	terminated	NCI	relapsed or refractory solid tumors, CNS tumors, lymphomas, or T-ALL
NCT01145456	I	**12** + gemcitabine	completed	NCI	advanced solid tumors
NCT01096355	I	**12**	completed	NCI	metastatic or nonremovable solid malignancies
NCT01232829	II	**12**	completed	NCI	metastatic pancreatic cancer
NCT00756717		**13** + tamoxifen or letrozole	ongoing	Loyola University; Merck Sharp & Dohme Corp.	breast cancer
NCT01193881	I	**12** + erlotinib	ongoing	NCI	stage IV or recurrent non-small cell lung cancer
NCT01270438	II	**12** + FOLFOX regimen + bevacizumab	withdrawn	NCI	metastatic colorectal cancer
NCT01149356	I	**12** + exemestane	terminated	NCI	advanced or metastatic breast cancer
NCT01175343	II	**12**	ongoing	NCI	recurrent and/or metastatic epithelial ovarian cancer, fallopian tube cancer, or primary peritoneal cancer
NCT01122901	II	**12**	ongoing	NCI	recurrent or progressive glioblastoma
NCT01071564	I	**12** + vismodegib	terminated	NCI	advanced breast cancer
NCT01218620	I	**12** + chemotherapeutics	completed	NCI	advanced solid tumors
NCT01119599	I	**12** + temozolomide + radiation therapy	ongoing	NCI	newly diagnosed malignant glioma
NCT00803894	I	**13**	completed	Merck Sharp & Dohme Corp	healthy male volunteers to identify biomarkers for Cancer or Alzheimer's disease
NCT01295632	I	**13** + ridaforolimus	ongoing	Merck Sharp & Dohme Corp	advanced cancer
NCT00572182	I	**13**	terminated	Pediatric Brain Tumor Consortium and NCI	CNS cancer
NCT00645333	I/II	**13** + docetaxel	completed	University of Michigan Cancer Center	advanced or metastatic breast cancer
NCT01243762	I	**13** + dalotuzumab	terminated	Merck Sharp & Dohme Corp	advanced cancer
NCT00106145	I	**13**	completed	Merck Sharp & Dohme Corp	metastatic or locally advanced breast cancer and other advanced solid tumors
NCT00828646	I	**14**	completed	Bristol-Myers Squibb	healthy volunteers
NCT01198535	I	**12** + cetuximab	terminated	NCI	metastatic colorectal cancer
NCT00100152	I	**13**	terminated	Merck Sharp & Dohme Corp	T-ALL
NCT01208441	I	**12** + letrozole	terminated	NCI	stage II/III hormone receptor-positive breast cancer
NCT01269411	I	**12**	terminated	NCI	recurrent malignant glioma
NCT01200810	II	**12** + bicalutamide	terminated	NCI	prostate cancer
NCT01070927	II	**12**	completed	Hoffmann-La Roche	recurrent or refractory nonsmall cell lung cancer
NCT00878189	I	**11**	ongoing	Pfizer	advanced solid tumors, T-ALL
NCT01057030	I	**14**	completed	Bristol-Myers Squibb	healthy young male subjects to assess safety
NCT01189240	I/II	**12** + bevacizumab	ongoing	NCI	progressive or recurrent malignant glioma
NCT01120275	II	**12**	ongoing	NCI	advanced melanoma
NCT00719394	I	**15**	completed	Wyeth/Pfizer	healthy male volunteers to assess safety
NCT01216787	II	**12**	withdrawn	NCI	stage IIIB, IIIC, or IV melanoma
NCT01192763	I	**12**	terminated	NCI	pancreatic cancer
NCT01193868	II	**12**	completed	NCI	advanced nonsmall cell lung cancer
NCT01158274	I	**12** + capecitabine	completed	NCI	refractory solid tumors
NCT01151449	II	**12**	ongoing	NCI	advanced, metastatic, or recurrent triple negative invasive breast carcinoma
NCT01198184	I	**12** + temsirolimus	completed	NCI	advanced solid tumors
NCT01217411	I/II	**12** + radiation therapy + stereotactic radiosurgery	terminated	NCI	brain metastases from breast cancer
NCT01141569	II	**12**	completed	NCI	advanced renal cell carcinoma
NCT01116687	II	**12**	completed	NCI	metastatic colorectal cancer

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Table 3. continued

NCT identifier	phase	therapeutic agent	status	sponsor/collaborators	tumor type
NCT01236586	I	**12**	withdrawn	NCI	children relapsed/refractory solid or CNS tumors, lymphoma, or T-ALL
NCT01098344	I	**13** + gemcitabine	completed	Cancer Research UK	stage III and IV pancreatic cancer

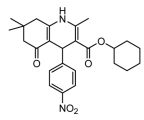
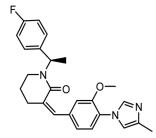
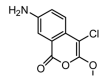
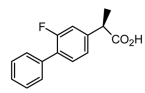
be challenged by other pan-GSIs but not by γ-secretase modulators. Furthermore, **14** blocks the binding of four different active site-directed GSI photoaffinity probes. Compound **14** reduces cerebrospinal fluid Aβ levels without causing Notch-related toxicity in rats and dogs, however, not all publications agree on that.[123] Gastrointestinal and dermatological manifestations, such as diarrhea, nausea, vomiting, rash, and itching skin, are the main side effects. GSI 136 (**15**, Table 1)[135,136] was developed by Wyeth (now a subsidiary of Pfizer) for AD treatment and evaluated in phase I clinical trials to determine its safety and tolerability in healthy subjects. The evaluation of this drug has been extended to other diseases. However, no information about the outcomes of the trials is presently available. GSI-953/begacestat (**16**, Table 1)[137] is an orally active GSI that selectively inhibits the cleavage of APP with an IC_{50} = 14.8 nM and is 16-fold more selective for APP over Notch (Notch: IC_{50} = 208.5 nM). LY-900009 (**17**, Table 1)[138] is a selective orally available small-molecule GSI inhibiting Notch activation with an IC_{50} = 0.27 nM; **17** was evaluated in clinical trials for the treatment of cancer. The study was terminated in Phase I due to adverse events, including diarrhea, vomiting, nausea, fatigue, anorexia, hypophosphatemia, and skin rash of different grades. As a GSI, **17** also decreases plasma levels of Aβ in a dose-dependent manner. BMS-299897 (**18**, Table 1)[139,140] is an orally active GSI with an IC_{50} = 12 nM for Aβ production.

Several other GSIs (Table 2) have been designed and evaluated only in experimental and preclinical settings, including a series of bicyclic sulfonamide pyrazoles[141] (structures not disclosed) (APP: IC_{50} = 0.4 nM; Notch: IC_{50} = 36 nM). These compounds may represent the next series of GSIs with clinical potential.

GSI-Based Clinical Trials. According to the role of the Notch pathway in cancer, several phase I clinical trials were initiated (Table 3, data obtained from ClinicalTrials.gov) in adult patients with advanced hematological and solid tumors to determine the maximum tolerated dose for further phase II trials and to assess safety, pharmacokinetics, and potential clinical activity. GSIs were tested either as single agents or in combination with a standard cancer chemotherapy regimen. From these early clinical studies, tolerability and toxicity have been acceptable in both single or combined therapies[151,152] with side effects comparable, but not worse, than standard chemotherapeutics. The main side effects encountered were gastrointestinal disorders. Some encouraging clinical benefits were observed associated with a modest decrease of the Notch gene signature. However, because only phase I or II studies are presently underway, it will be necessary to wait for the results of phase III trials of large multicenter cohorts of cancer patients to determine possible clinical benefits of GSIs.

Non-GSI-Based Therapeutics. Non-GSI small molecule inhibitors have also been evaluated in preclinical settings, which include γ-secretase modulators[153] like E2012/HY10016[154] (**23**, Table 4) and JLK6[155] (**24**, Table 4) and nonsteroidal anti-inflammatory drugs (NSAID) like flurbiprofen/flurizan[156] (**25**, Table 4) or the endoplasmic reticulum-exporting inhibitor molecule **6**.[90] Their modes of biological action are presently not well-understood, but γ-secretase modulators and nonsteroidal anti-inflammatory drugs are hypothesized to modulate the enzyme hydrolytic step, thus altering its cleavage site preference depending on the γ-secretase complex expressed. However, these drugs have high IC_{50} values and seem to be more efficient at inhibiting APP processing than the other potential γ-secretase substrates, including Notch. A deeper understanding of their modes of action may be helpful in designing substrate-specific inhibitors of γ-secretase.

Table 4. Synthetic Notch Antagonists with Non-GSI Modes of Action

code	*structure*	*IC_{50}*	*comments*	*reference*
6 FLI-06		Notch: 2.3 μM	inhibits ER exporting	90
23 E2012		Aβ:0.15-5.6 μM	benzazepine no effects on Notch processing; inhibits 3β-hydroxysterol Δ24-reductase in the cholesterol biosynthesis	154
24 JLK6		Aβ:30 μM	isocoumarin no effects on Notch cleavage	155
25 Flurizan Flurbiprofen		Aβ active 100 μM	nonsteroidal anti-inflammatory drug (NSAID) inhibits γ-secretase	156

More recently, bioengineered antibodies or antibody−drug conjugates[157,158] targeting Notch receptors, Notch ligands, Notch receptor−ligand interactions, or γ-secretase itself have also been developed. Some of them are presently being evaluated in clinical trials (Table 5).

Clinical Trials Evaluating ADAM Inhibitors. Therapeutic options other than targeting the γ-secretase−Notch pathway involve targeting the other Notch-activating enzymes, either the furins or the ADAM10 and ADAM17 proteases. We do not believe that targeting the intracellular multipurpose furins is a viable option. Targeting the cell-surface ADAMs may be more interesting.[116,159] The matrix metalloproteinases (MMPs) were linked to cancer progression many years ago, and their potential value as therapeutic targets for cancer has been examined in numerous clinical trials, unfortunately with mostly negative results. These previous trials used broad-spectrum inhibitors, which was hypothesized to be the cause of failure. More selective inhibitors for ADAM10 or ADAM17 have been designed and developed[160] and are presently being evaluated for clinical use in a specific cancer, diffuse large B cell

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Table 5. Ongoing and Completed Clinical Trials of Antibodies Targeting Notch Signaling

NCT identifier	phase	therapeutic agent	status	sponsor/collaborators	tumor type
NCT01577745	I	MEDI0639 (anti-DLL-4 mAb)	recruiting	MedImmune LLC	advanced solid tumors
NCT00744562	I	demcizumab (anti-DLL-4 mAb)	completed	OncoMed Pharmaceuticals, Inc.	solid tumors
NCT00871559	I	REGN421 (SAR153192)	completed	Regeneron Pharmaceuticals and Sanofi	advanced solid malignancies
NCT02298387	I	OMP-305B83 anti-DLL-4/VEGF bispecific mAb	recruiting	OncoMed Pharmaceuticals, Inc.	solid tumors
NCT01189942	I	demcizumab + folinic acid + fluorouracil + irinotecan	ongoing	OncoMed Pharmaceuticals, Inc. and Novotech (Australia) Pty Limited	metastatic colorectal cancer
NCT01189968	I	demcizumab + carboplatin + pemetrexed	recruiting	OncoMed Pharmaceuticals, Inc. and Novotech (Australia) Pty Limited	non-small cell lung cancer
NCT01189929	I	demcizumab + abraxane + gemcitabine	recruiting	OncoMed Pharmaceuticals, Inc. and Novotech (Australia) Pty Limited	locally advanced or metastatic pancreatic cancer
NCT01952249	I/II	demcizumab + paclitaxel	recruiting	OncoMed Pharmaceuticals, Inc.	ovarian, primary peritoneal, or fallopian tube cancer
NCT01703572	I	OMP-52M51 (anti-Notch 1 mAb)	recruiting	OncoMed Pharmaceuticals, Inc. and GlaxoSmithKline	lymphoid malignancies
NCT01778439	I	OMP-52M51	recruiting	OncoMed Pharmaceuticals, Inc. and GlaxoSmithKline	solid tumors
NCT01647828	I/II	OMP-59R5 anti-Notch 2/3 mAb + gemcitabine + Nab-paclitaxel	recruiting	OncoMed Pharmaceuticals, Inc.	pancreatic cancer
NCT01859741	I/II	OMP-59R5 + etoposide + cisplatin or carboplatin	recruiting	OncoMed Pharmaceuticals, Inc.	small cell lung cancer
NCT01277146	I	OMP-59R5	ongoing	OncoMed Pharmaceuticals, Inc.	solid tumors
NCT01013597	II	LBH589 (panobinostat, a selective HDAC inhibitor)	ongoing	University of Wisconsin, Madison and Novartis Pharmaceuticals	metastatic thyroid cancer
NCT00985946	II	LBH589	ongoing	University of Wisconsin, Madison and Novartis Pharmaceuticals	neuroendocrine tumors

Table 6. Clinical Trials Evaluating ADAM Inhibitors

compound	NCT identifier	phase	therapeutic agent	status	sponsor/collaborators	tumor type
ADAM17 inhibitor	NCT02141451	I/II	INCB7839	recruiting	Masonic Cancer Center, University of Minnesota	diffuse large B cell lymphoma
ADAM10/17 inhibitor	NCT00312780	II	XL784	completed	Symphony Evolution, Inc.	diabetic nephropathy

lymphoma, as well as in inflammatory diseases and diabetic nephropathy (Table 6).

Presently, only very few reports evaluating ADAM inhibition on the Notch pathway have been published. A recent study[161] comparing ADAM17 inhibition and **1**-mediated γ-secretase inhibition has shown an advantage for ADAM inhibition in a model of renal carcinoma; unfortunately, these authors used Marimastat as the ADAM inhibitor, which can also inhibit other MMPs in addition to ADAM17. To the best of our knowledge, no clinical trials evaluating such inhibitors in the Notch context are underway, whereas ADAM inhibitors are clinically tested for cancer and inflammatory diseases. Whether in the long term a role for regulating the Notch pathway using these inhibitors will come to light requires more detailed analysis of these trials.

9. THE PROBLEM OF SELECTIVITY/SPECIFICITY OF γ-SECRETASE INHIBITION

The major challenge in targeting the Notch pathway for therapeutic purposes is both selectivity and specificity of the drugs aimed at controlling Notch functions both in cancer and in inflammatory diseases. The use of drugs designed and developed for inhibiting γ-secretase has been the first therapeutic attempt to control the Notch signaling pathway. However, clinical trials with these drugs have generally resulted in toxicities and side effects attributed to the inhibition of hydrolysis of AD-unrelated substrates of this protease, including Notch. Therefore, more tissue-, cell-, and Notch-selective therapeutic tools must be designed for the treatment of cancer or inflammatory diseases.

In a recent approach,[162] using experimental rodent models of inflammatory and profibrotic kidney diseases and toward selective targeting of GSIs, we have developed prodrug strategies as potentially selective, precise, and efficient systems. To achieve disease selectivity of GSIs, we have designed and evaluated pro-drugs of GSI RO5016025[162] (**26**, Figure 5), a close analogue of **12**, as substrates for enzymatic activities specifically expressed in diseased kidneys. Previous data had shown that the activities of the peptidases aminopeptidase A (APA, substrate: α-Glu-XX), γ-glutamyltransferase (γ-GT, substrate: γ-Glu-XX), and γ-glutamylcyclotransferase (γ-GCT, substrate: N-Ac-γ-Glu-XX) are highly increased in tissues from

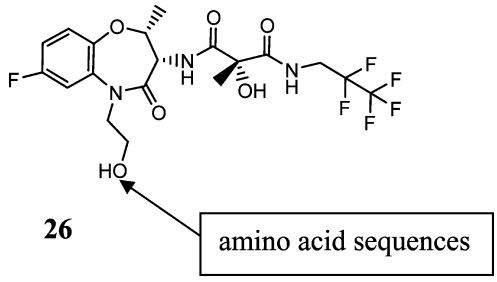

Figure 5. Chemical structure of a protease-targeted pro-drug of a γ-secretase inhibitor.

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patients and rodent experimental models of human diseases, including kidney diseases, suggesting that these enzymes may be used for drug targeting purposes. We therefore developed γ-secretase inhibitor-based prodrugs as potential substrates for γ-GT, γ-GCT, and APA (Figure 5), and in vitro and in vivo evaluation demonstrated the interest of using γ-Glu prodrugs but not α-Glu prodrugs of GSIs.

10. CONCLUSIONS AND PERSPECTIVES

The canonical Notch pathway with four receptors and five ligands plays critical roles in context-dependent tissue and cell-fate determination. In cancer, this pathway has a determining impact on tumor behavior and response to therapy. Considering the role of Notch signaling in the pathogenesis of different cancers, Notch proteins in general function as oncogenes; however, tumor suppressive functions have also been reported in certain cell types, highlighting the context-dependent role of Notch signaling. Presently, no Notch-targeting therapeutics are FDA-approved, but multiple therapeutics are under clinical evaluation for cancer, including GSIs and synthetic or non-GSI biological molecules, such as bioengineered antibodies or antagonists.[162,163] No large multicenter phase III clinical trial has yet been initiated; therefore, it is premature to foresee the future of these therapeutics, but the tolerability profile has been shown to be acceptable (at least in the context of cancer) in early phase I/II clinical trials.

However, because of the potential side effects of these drugs, more cell- and tissue-selective and specific inhibitors are needed. The most studied target of the Notch pathway, the activating enzyme γ-secretase, is an intramembrane protein complex with multiple potential and actual substrates identified. Nonselective inhibition of γ-secretase by the initial GSIs has been shown to result in serious side effects mainly due to interfering with the normal functions of Notch as well as the other substrates of γ-secretase. A deeper understanding of the enzymatic mechanisms and mode of binding of its substrates and modulators[163−168] may lead to the development of more cell- and tissue-selective and specific Notch inhibitors for the therapy of cancer or inflammatory diseases. GSIs able to inhibit Notch cleavage with slightly improved selectivity over the other biological substrates of this protease are being developed and must be evaluated in clinical trials. A second attempt has been to develop antibodies to inhibit the binding of ligands to their Notch receptors or to inhibit γ-secretase enzyme activity. These antibodies are also presently under clinical evaluation, mainly for cancer.[169] A different approach involves the development of targeting prodrugs of GSIs as potentially selective, precise, and efficient systems, using overexpressed diseased tissue-selective molecules, thus increasing local drug distribution and decreasing toxicity and off-target effects.[162] A yet unexplored (to the best of our knowledge) therapeutic approach involves the use of nanoparticles loaded with Notch antagonistic drugs. In most normal blood vessels, the gaps between vascular cells are too small to allow for the passage of nanoparticles. Both cancer and inflammatory diseases result in impaired vascular function and leaky blood vessels, the enhanced permeability and retention (EPR) effect, allowing the passage of nanoparticles across the disease-associated vascular wall into the organs and their retention in the organs. Other possibilities involve targeting the other Notch-activating enzymes, either the furin-like proteases or the ADAM10 and ADAM17 proteases. We do not believe that targeting the intracellular multipurpose furins is a viable option. Targeting the cell-surface ADAMs may be an option to consider for future therapeutic developments in inflammatory and oncologic diseases, however, with problems of selectivity and specificity of ADAM inhibition for the diseased tissues comparable to γ-secretase inhibition.

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Notes

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ABBREVIATIONS USED

Aβ: amyloid-beta peptides; AD: Alzheimer's disease; APP: amyloid precursor protein; ADAM: a disintegrin and metalloproteinase; APA: aminopeptidase A; DLL: Delta-like ligand; EGF: epidermal growth factor; EMT: epithelial-to-mesenchymal transdifferentiation; EPR: enhanced permeability and retention; GSI: γ-secretase inhibitor; γGT: γ-glutamyl transferase; γGCT: γ-glutamyl-cyclotransferase; mAb: monoclonal antibody; NICD: Notch intracellular domain; PACE: paired basic amino acid cleaving enzyme; PS: presenilin; shRNA: small interfering RNA; T-ALL: T-cell acute leukemia; TGFβ: transforming growth factor-beta; TIF: tubullo-interstitial fibrosis; VEGF: vascular endothelial growth factor

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