

Published in final edited form as:

Pharmacol Ther. 2013 August ; 139(2): 95–110. doi:10.1016/j.pharmthera.2013.02.003.

Notch Inhibitors for Cancer Treatment

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Abstract

Notch signaling is an evolutionarily conserved cell signaling pathway involved in cell fate during development, stem cell renewal and differentiation in postnatal tissues. Roles for Notch in carcinogenesis, in the biology of cancer stem cells and tumor angiogenesis have been reported. These features identify Notch as a potential therapeutic target in oncology. Based on the molecular structure of Notch receptor, Notch ligands and Notch activators, a set of Notch pathway inhibitors have been developed. Most of these inhibitors had shown anti-tumor effects in preclinical studies. At the same time, the combinatorial effect of these inhibitors with current chemotherapeutic drugs still under study in different clinical trials. In this review, we describe the basics of Notch signaling and the role of Notch in normal and cancer stem cells as a logic way to develop different Notch inhibitors and their current stage of progress for cancer patient's treatment.

Keywords

Notch signaling; Notch inhibitors; cancer; cancer stem cells

1. Introduction

Targeted therapies have emerged over the last decade as a new and stimulating novelty strategy for cancer treatment. Nevertheless, to develop and validate a targeted molecule takes some time. However, the development and validation of these agents requires significant investment in cancer biology. The first step is to identify a molecular target marker that is crucial for cancer cell proliferation and survival, ideally one that is expressed/repressed preferentially or specifically in the malignant cell tissue. The following step is to develop a targeting strategy based on the structure and function of the putative target. This may be relatively simple in the case of a kinase, and more complex in cases like adaptor proteins or non-kinase receptors. generate and develop a therapeutic agent that target specifically or mostly this molecular target. In this step, understanding the structure, function and post-translational modifications of the candidate target, including cross-talk with other druggable targets, are essential to develop a practical targeting strategy. protein structure plays a crucial role to identify the domains or fragments of these proteins that are potentially "druggables". In this context, Notch receptors and other components of the

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6. Conflict of interest/disclosures

No conflict of interest.

Notch signaling pathway appear to be goodare potentially attractive therapeutic targets. In this review we will describe the modular structure of Notch receptor and Notch ligands, the effects of the their aberrant expression or dysregulated function of them in cancer and cancer stem cells, and the targeted therapeutic tools developed to inhibit block the activation of the Notch signaling pathway based oin our current understanding of their molecular structures and post-translational modifications.

1.1 Notch receptors

1.1.1 Structure—The Notch receptor is a single pass trans-membrane protein evolutionarily conserved from sea urchins to humans. It contains an extracellular domain (N^{EC}), a transmembrane domain (N^{TM}) and an intracellular domain (N^{IC}). The extracellular subunit of Notch possesses a multi-modular structure containing multiple Ca^{2+} binding epidermal growth factor-like repeats (EGF-like) that are required for ligand interaction (Rebay et al., 1991), a negative regulatory region (NRR) which is composed of three cysteine-rich Lin12/Notch repeats (LN) each containing a Ca^{2+} binding site (Aster et al., 1999; Gordon et al., 2007) and a C-terminal hydrophobic region. The LN repeats stabilize the interaction between subunits by preventing ligand-independent cleavage by metalloproteases (Sanchez-Irizarry et al., 2004). As the name suggests, the NRR holds the mature Notch heterodimer in an auto-inhibited state. The transmembrane subunit includes a short extracellular region containing a pair of conserved cysteines (Kidd et al., 1986; Mumm et al., 2000) thought to participate in heterodimerization (Weinmaster et al., 1992), a Type I transmembrane region and an intracellular region that contains a RBP-jk association module (RAM) that interacts with its transcriptional coactivator RBP-Jk or CSL (CBF-1/Suppressor of Hairless/LAG1) (Tamura et al., 1995). The RAM domain is followed by seven ankyrin (ANK) repeats (Lubman et al., 2004) that interact with CSL and other transcriptional regulators (Nam et al., 2006), two nuclear-localization signals (NLSs) (Lieber et al., 1993), a transactivation domain (TAD) (Kurooka et al., 1998) which ends in a polyglutamine stretch (OPA) (Kurooka et al., 1998) and a C-terminal PEST sequence (a region rich in Proline, Glutamic acid, Serine, and Threonine) that contains multiple phosphorylation sites, which are important for the control of N^{IC} stability and serve as triggers for subsequent ubiquitination and turnover of the receptor (Rechsteiner, 1988).

While *Drosophila* has only one Notch gene, the mammalian Notch family consists of four members (Notch1, 2, 3, and 4) that are approximately 60% homologous to each other and to *Drosophila* Notch (Lardelli et al., 1995; Callahan et al., 2001). Although the overall structure of Notch receptors is similar, there are significant differences. The Notch1 and Notch2 receptors contain 36 EGF repeats (Weinmaster et al., 1992; del Amo et al., 1993) in their extracellular domains, similar to *Drosophila*; whereas Notch3 contain 34 repeats (Lardelli et al., 1994) and Notch4 contains 29 repeats (Uyttendaele et al., 1996). The other difference is in the transactivation domain. Notch1 and Notch2 contain a strong and a weak TAD, respectively (Kurooka et al., 1998), Notch3 has a potent but specific TAD best suited to the activation of the *hes5* promoter (Ong et al., 2006). In contrast, Notch4 does not contain a TAD (Fig. 1A–B). These structural differences may offer clues to the functional divergence among mammalian Notch paralogs.

1.1.2 Post-transcriptional modifications of Notch Receptors—Increasing number of reports have shown that N^{IC} is subject to a variety of post-translational modifications that regulate Notch activity. These modifications include glycosylation, ubiquitylation, phosphorylation, acetylation and hydroxylation.

1.1.2.1 Glycosylation: Glycosylation of Notch receptors by Fringe enzymes (*N*-acetylglucosaminidyltransferases) affects binding affinities between ligands and specific

EGF-repeats (Okajima et al., 2003). Fringe glycosyl transferases initiate elongation of O-linked fucose residues on specific EGF-like repeats of Notch receptors (Bruckner et al., 2000; Moloney, Panin, et al., 2000; Moloney, Shair, et al., 2000). This modification prevents Notch activation by Jagged ligands, but not by Delta-like ligands (Panin et al., 1997). In *Drosophila*, glycosyltransferase RUMI, also modifies Notch by adding O-glucose to serine residues on particular Notch consensus sequences (Acar et al., 2008) but the importance of this modification in mammals remains to be demonstrated. In mammals three Fringe genes are known, Lunatic Fringe (*Lfng*), Manic Fringe (*Mfng*), and Radical Fringe (*Rfng*) (Cohen et al., 1997). Reduced *Lfng* expression has been recently demonstrated in basal-like triple-negative breast cancer (TNBC). Importantly, targeted deletion of *Lfng* in the mouse mammary gland induces TNBC-like mammary cancers with high expression of cleaved Notch receptors. In this model, *Lfng* blocked mammary stem cells proliferation (Xu et al., 2012).

1.1.2.2 Ubiquitylation: Monoubiquitination has been proposed to result in Notch activation (Gupta-Rossi et al., 2004). Conversely, polyubiquitination can lead to downregulation of Notch signaling. The Ring Finger E3 ubiquitin ligase Deltex along with β -arrestin/Kurtz (Mukherjee et al., 2005), E3 ubiquitin ligases Itch/AIP4 (Atrophin-1 interacting protein 4) (Qiu et al., 2000), NEDD4 (neural precursor cell expressed developmentally down-regulated 4) (Sakata et al., 2004) and Cbl (Casitas B-lineage lymphoma) (Jehn et al., 2002) can polyubiquitinate Notch in the cytoplasm and direct Notch receptor via endocytosis towards lysosomal degradation or toward recycling to the plasma membrane (Nichols et al., 2007). Several E3 ubiquitin ligases including Fbw7/Sel-10 (Oberg et al., 2001), Itch (Qiu et al., 2000), c-Cbl (Jehn et al., 2002), and Deltex (Mukherjee et al., 2005) can ubiquitinate active Notch and target it to the proteasome for degradation. Endocytosis can sort Notch to either activation (see above) or degradation pathways. Numb is a cytoplasmic negative regulator of Notch. Numb, in cooperation with the AP2 (adaptor protein-2) component α -adaptin promotes Notch endocytosis (Santolini et al., 2000) followed by proteasome-mediated degradation (McGill et al., 2003). Prolyl isomerase Pin-1 can modify N^{IC}, increasing its intracellular half-life (Rustighi et al., 2009). Pin-1 in turn is regulated by mixed lineage kinases (MLK), potentially placing this pathway upstream of Notch (Rangasamy et al., 2012).

1.1.2.3 Phosphorylation: The N^{IC} is phosphorylated by several kinases at different residues. Phosphorylation of N^{IC} by glycogen synthase kinase 3 β (GSK3 β) occurs C-terminally to the ANK repeats and inhibits Notch2^{IC}-mediated induction of genes such as hairy and enhancer of split 1 (Hes1), but stabilizes N^{IC} (Foltz et al., 2002). Granulocyte colony stimulating factor (Csf) also induces phosphorylation of Notch2^{IC}, leading to its inactivation (Ingles-Esteve et al., 2001). Cyclin C/cyclin-dependent kinase (CDK) 8 phosphorylates Notch^{IC}, and this modification is important for both the activity and turnover of N^{IC} (Fryer et al., 2004).

1.1.2.4 Acetylation: Acetylation controls the stability of N^{IC} (Popko-Scibor et al., 2011; Palermo et al., 2012). The deacetylase Sirtuin 1 (SIRT1) has been reported as a key regulator of the endothelial Notch signaling (Guarani et al., 2011).

1.1.2.5 Hydroxylation: It has been described that the asparagine hydroxylase factor-inhibiting HIF1 α (FIH1 α), which also operates in the cellular hypoxic response, hydroxylates N^{IC} at two residues (N1945 and N2012) (Coleman et al., 2007; Zheng et al., 2008). Interestingly, Notch1^{IC}, 2^{IC} and 3^{IC}, but not Notch4^{IC}, are hydroxylated by FIH1 α , and this might contribute to differential signaling. *In vitro* data suggest that FIH1 negatively regulates Notch signaling, but the biological significance of the FIH1-mediated

modifications is not fully understood, and mice targeted for FIH1 do not display an overt Notch gain-of-function phenotype (Zhang et al., 2010)

1.2 Notch ligands

Drosophila has 2 canonical ligands, Delta and Serrate. Mammals express five canonical Notch ligands: three are homologous to Delta and are named Delta-like-1, -3 and -4 (DLL1, DLL3 and DLL4) and two are homologous to Serrate and are named Jagged1 and Jagged2 (Lindsell et al., 1995; Shawber et al., 1996; Dunwoodie et al., 1997; Shutter et al., 2000). These ligands are Type I single-pass transmembrane proteins with an extracellular region consisting of an N-terminal region, a cysteine-rich DSL (an acronym for Delta, Serrate and LAG-2) motif and varying number of EGF-like repeats, similar to the Notch proteins (Kopan et al., 2009). The N-terminal region, the DSL domain and the first two EGF-like repeats are necessary for interaction with EGF repeats 11 and 12 of Notch receptors (Shimizu et al., 1999; Parks et al., 2006). The intracellular regions of DSL ligands are not conserved, but some contain multiple lysine residues and a C-terminal PDZL (PSD-95/Dlg/ZO-1 ligand) motif involved in the ligand signal activity and interactions with the cytoskeleton (Pintar et al., 2007). Notch signaling can also be activated by “non-canonical” ligands other than Delta/Jagged, such as F3/contactin (Hu et al., 2003), DLK1 & 2, DNER, and EGFL7 (Schmidt et al., 2009; D'Souza et al., 2010) (Fig. 1A–B).

The structural variability observed in mammals among the four Notch proteins and their differential context-dependent functions open the possibility of specific targeting with monoclonal antibodies (mAbs) against the least conserved regions of the proteins.

2. Notch signaling pathway

2.1 Canonical Notch signaling pathways

Most of our information on the canonical Notch signaling pathway is derived from studies on Drosophila Notch and its mammalian orthologue Notch1. The Notch precursor protein is produced as a single-chain transmembrane protein in the endoplasmic reticulum where it interacts with O-fucosyltransferase 1 (OFUT1 in Drosophila, POFUT1 in mammals) (Okajima et al., 2005). It is then transported to the Golgi where it is cleaved by a Furin-like convertase at site 1 (S1) (Logeat et al., 1998) and glycosylated by OFUT (Okajima et al., 2002; Shi et al., 2003) and Fringe family N-acetylglucosaminidyl transferases (Haines et al., 2003). Cleaved, glycosylated Notch is transported to the cell surface as a mature heterodimer.

At the plasma membrane, Notch signaling is initiated by a Notch receptor-ligand interaction between two neighboring cells, which induces two successive proteolytic cleavages within the NTM subunit that are required to release the intracellular fragment of Notch (N^{IC}) from the membrane (Mumm et al., 2000). The interaction between Notch and its ligand DSL in *trans* generates an activating interaction on neighboring cells. In contrast, inhibitory *cis* interactions between receptor and ligand in the same cell suppress Notch signaling (de Celis et al., 1997; Li et al., 2004; Miller et al., 2009). The *cis* interaction between Notch and DSL is thought to be bidirectional (Becam et al., 2010; Sprinzak et al., 2010). Ubiquitin ligases Mindbomb (Itoh et al., 2003) or Neuralized (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001) interact with the intracellular domains of ligands to promote ligand ubiquitination and internalization by transendocytosis to the ligand-expressing cell (Parks et al., 2000). This endocytic process may be required to generate sufficient mechanical force to disrupt the hydrophobic interactions between the N-terminal portion of NTM and the C-terminal portion of N^{EC} (Tianont et al., 2011). Subunit dissociation exposes a cleavage site (S2) on NTM on the extracellular side of the membrane for A Disintegrin And Metalloprotease 10 (ADAM10) or 17 (ADAM17) (Brou et al., 2000; Mumm et al., 2000).

ADAM cleavage produce a short extracellular truncation fragment, and a clipped transmembrane spanning intermediate called NEXT (Notch Extracellular Truncation) which serves as a substrate for the final proteolytic cleavage (Saxena et al., 2001). The latter occur at site 3 (S3)(Schroeter et al., 1998) and site 4 (S4) within the transmembrane domain and are mediated by the γ -secretase activity of a multi-protein complex consisting of four subunits, presenilin 1 or 2 (the catalytic subunit, an aspartyl protease) (Chen et al., 2006), nicastrin (which maintains complex stability and regulates intracellular protein trafficking) (Zhang et al., 2005), APH1 (anterior pharynx-defective 1; required for the proteolytic activity) (Lee et al., 2004) and PEN2 (presenilin enhancer 2; stabilizes the complex after presenilin proteolysis has generated the activated N-terminal and C-terminal fragments) (Prokop et al., 2004). γ -Secretase cleavage and release of N^{IC} can occur at the cell surface or in an endosomal compartments, but cleavage at the membrane is thought to produce a more stable form of N^{IC} (Tagami et al., 2008; Kopan et al., 2009). After cleavage, N^{IC} translocates to the nucleus where it binds to its downstream transcription factor CSL and drives canonical Notch-mediated gene transcription (Fortini et al., 1994). CSL is thought to be bound to target DNA in a repressive complex that contains histone deacetylases (Lai, 2002), co-repressors SMRT (silencing mediator for retinoid and thyroid receptor)/N-CoR (nuclear receptor co-repressor) (Kao et al., 1998), CIR (CSL interacting repressor) (Hsieh et al., 1999) and SHARP (SMRT/HDAC-1-associated repressor protein)/MINT/SPEN (Oswald et al., 2002; Oswald et al., 2005). N^{IC} competes with the co-repressor complex to bind to CSL and interacts first through its RAM domain (Nam et al., 2006). The ANK domain then associates with CSL to recruit the coactivator Mastermind-like 1 (MAML1, one of three mammalian MAML homologues of Drosophila Mastermind or MAM) (Wu et al., 2000). The Notch-CSL-MAML1 ternary complex in turn recruits other coactivators like histone acetyltransferases CBP/p300 (Wallberg et al., 2002) or PCAF/GCN5 (Kurooka et al., 2000), which convert CSL from a transcriptional repressor to a transcriptional activator. Crystallographic data have shown that the ankyrin domain of N^{IC} and the N- and C-terminals of the Rel homology domain of CSL form a complex with the long, kinked N-terminal helix of MAML1 (Nam et al., 2006). In this complex, the relatively unstructured N-terminal region of the ANK domain, which includes the RAM sequence, folds to form the N-terminal ANK repeat, creating a 7-repeat domain. CSL-binding sites on some Notch promoters exist in pairs in a head-to-head arrangement and could recruit dimeric Notch transcription complexes (Nam et al., 2007) which could increase the strength of the Notch signal. The end result of canonical Notch activation is transcriptional de-repression of a group of genes, many of which are themselves transcription factors or transcriptional repressors. This generates a cascade of gene regulatory events that can modulate virtually every aspect of cell fate decisions depending on cellular context. Recent CHIP-Seq data (Cho et al., 2011; Wang, Zou, et al., 2011; Zhao et al., 2011) have started to shed light on factors contributing to “cellular context”, at least in T- and B-lineage cells. In T-ALL (T-lymphoblastic leukemia) cells, ETS and RUNX family factors are frequently bound to chromatin close to CSL, and appear to cooperate with Notch/CSL, consistent with their known roles in T-cell development and Notch signaling. CREB also appears to cooperate with Notch/CSL at low affinity CSL sites. Zinc finger protein ZNF143 may control the accessibility of CSL to Notch/CSL complexes. ZNF143 sites were predominantly associated with repressive chromatin marks, as were CSL-only sites that contained CSL but not Notch. In proliferating lymphoblastoid cells (LCLs) expressing EBNA2, Wang et al. (Wang, Zou, et al., 2011) found that EBNA2 and CSL bind predominantly at nonpromoter sites. EBF, ETS, RUNX, PU.1, and NF- κ B (RELA) sites were found within 500bp of CSL sites. This correlated strongly with actual occupancy data for these transcription factors. Thus, the choice of genomic CSL sites at which Notch activates transcription may depend, at least in part, on the presence of additional transcriptional regulators that can cooperate with or antagonize the Notch-CSL transcriptional complexes. Different cells or different cellular states may have a variety of Notch target sites based on similar mechanisms. “Classical” Notch target genes

include among others nuclear basic helix-loop-helix proteins (bHLH) of the Hairy/Enhancer of Split family (HES1–5) (Lecourtois et al., 1995), the Hairy-related family (HRT) (Nakagawa et al., 2000), and the Hairy/Enhancer of Split-related with YRPW motif (HEY) families (Maier et al., 2000). These negatively modulate the expression of genes such as the Achaete-Scute family that induce neuronal differentiation. N^{IC} is also thought to upregulate Deltex (Choi et al., 2002), several members of the NF- κ B family, at least in bone marrow hematopoietic cells, (Oswald et al., 1998; Cheng et al., 2001), PPAR family transcription factors (Nickoloff et al., 2002), as well as cell cycle regulators p21WAF1-CIP1 (Rangarajan et al., 2001), cyclin D1 (Ronchini et al., 2001), and c-Myc (Weng et al., 2006). Recently, the Notch1^{IC} nuclear interactome has been characterized in T-ALL cells (Yatim et al., 2012). In these cells, N^{IC} interacts with numerous transcriptional coactivators, and assembles a multifunctional complex containing AF4p12, the PBAF/BRG1 nucleosome remodeling complex, and histone demethylases LSD1 and PHF8 (Yatim et al., 2012). Knockdown experiments showed that these factors regulate the expression of Notch-target genes. Additionally, Notch1^{IC} was shown to interact with multiple proteins involved in trafficking, signal crosstalk, post-translational modifications, DNA repair/replication and RNA processing. The functional significance and context-specificity of these interactions remain to be determined, but some of these may relate to non-canonical Notch signaling.

2.2 Non-canonical Notch signaling pathways

These pathways have been characterized as signals that respond to Notch independently of CSL (Type I), signals that activate Notch independently of S3 cleavage (Type II), or signals that activate CSL-dependent genes without Notch cleavage and N^{IC} release (Type III) (Sanalkumar et al., 2010). Among suggested mechanism of non-canonical Notch signaling are interactions of Notch with non-CSL transcription factors, such as β -catenin (Hayward et al., 2005), HIF-1a (hypoxia-inducible factor-1a) (Gustafsson et al., 2005), NF- κ B ((Guan et al., 1996), reviewed in (Osipo, Golde, et al., 2008)), and estrogen receptor ER α (Hao et al., 2010). Non-canonical Notch functions have mostly been identified in stem/progenitor cells or embryonic/primordial cells across species which are capable of expansion and/or differentiation. This suggests that non-canonical Notch signals might play an important role in undifferentiated early cell populations and might interact with conserved cell regulators.

While canonical Notch ligands are responsible for the majority of Notch signaling, a diverse group of structurally unrelated non-canonical ligands has also been identified that activate Notch and likely contribute to the pleiotropic effects of Notch signaling. Thus the final biological effects of Notch targeting are difficult to predict, and depend both on the involved ligand(s) and receptors, and signaling through the canonical intracellular pathway is modulated by non-canonical signaling.

2.3 Crosstalk between Notch and other oncogenic pathways

Numerous oncogenic pathways that cross-talk with Notch signaling have been described. Notch1 is required for the transforming activity of H-Ras (Weijzen et al., 2002) and TGF- α (Miyamoto et al., 2003). Notch activates the PI3K–AKT pathway (Liu et al., 2006; McKenzie et al., 2006; Bedogni et al., 2008; Calzavara et al., 2008; Graziani et al., 2008; Katoh et al., 2009; Yao et al., 2010; Cornejo et al., 2011; Guo, Teng, et al., 2011) while the AKT pathway upregulates Notch1 in response to VEGF (vascular endothelial growth factor) (Liu et al., 2003). Both the AKT and ERK pathways cooperate with Notch4 in transforming breast epithelial cells (Fitzgerald et al., 2000). Glycogen synthase kinase 3 beta (GSK3 β), which is negatively regulated by AKT, decreases the half-life of Notch (Foltz et al., 2002). Our group and others have demonstrated that in ER α -positive breast cancer cells estrogen causes accumulation of Notch1 (Soares et al., 2004; Rizzo, Miao, et al., 2008). We also demonstrated that this Notch1 accumulation occur at the plasma membrane (inactive form)

and that estrogen also inhibits Notch signaling while estrogen deprivation reactivates this pathway (Rizzo, Miao, et al., 2008). Similarly, Calaf et al. have shown that estrogen can promote malignant transformation of an immortalized human breast epithelial cell line, MCF-10F, increasing the expression of cell adhesion proteins such as Notch (Calaf et al., 2008). We also reported that HER2/neu overexpression inhibits Notch signaling while downregulation of HER2/neu or inhibition of its signaling caused reactivation of Notch signaling (Osipo, Patel, et al., 2008). Recently, Clementz et al. demonstrated that Notch1 and Notch4 are transcriptional targets of PEA3 (Clementz et al., 2011), a transcription factor whose expression has been associated with the malignant phenotype (Trimble et al., 1993; Shepherd et al., 2001) and with HER2/neu expression in breast carcinoma (Benz et al., 1997), and predicted worse overall survival in this malignancy (Kinoshita et al., 2002). Targeting PEA3 may indirectly inhibit Notch pathways, and provide a new therapeutic strategy for triple-negative and possibly other breast cancer subtypes (Clementz et al., 2011). Finally, we and others (Aguilera et al., 2004; Fernandez-Majada et al., 2007; Song et al., 2008; Hao et al., 2010) have described physical interactions between Notch1^{IC} and the IKK signalosome or nuclear IKK α . These effects are suggested to mediate Notch-induced activation of NF- κ B (Song et al., 2008) and ER α (Hao et al., 2010). Notch3^{IC} has also been shown to bind IKK α homodimers, resulting in activation of the NF- κ B alternative pathway (Barbarulo et al., 2011). Conversely, nuclear IKK α has been shown to activate Notch-dependent transcription in colon cancer cells (Fernandez-Majada et al., 2007). Recently, Guo and Gonzalez-Perez showed a new Notch, IL-1, and leptin crosstalk. They demonstrated that the leptin proangiogenic effects, via upregulation of VEGF/VEGFR-2, are mediated by leptin-induced Notch expression in breast cancer (Guo and Gonzalez-Perez, 2011).

In summary, Notch is the nexus of a unique and versatile signaling network that regulates and is regulated by a variety of cellular mechanisms highly dependent on cellular context. Thus, therapeutic targeting of the Notch pathway presents both promise and challenges. Successful development of Notch-targeting agents will require a mechanistic understanding of the role of Notch in specific diseases, and ideally, mechanism-based combination regimens. (Fig. 2)

2.4 Notch signaling and cancer

Notch was first identified as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL) in which a t(7;9) chromosomal translocation fuses the N-terminal region of the T-cell receptor beta (TCR β) to the C-terminus of Notch1 (Ellisen et al., 1991). This leads to expression of a truncated Notch1 protein lacking the extracellular subunit and hence constitutively active (Greenwald, 1994). It was later discovered that over 50% of T-ALL have a variety of mutations that activate Notch1 (Weng et al., 2004). These mutations are concentrated in the heterodimerization region, leading to destabilization of the interaction between the two subunits, and/or in the C-terminal PEST region and prolongation of the intracellular half-life of Notch. Further, loss of the E3 ubiquitin ligase Fbw27/Sel-10, or mutations that target the Fbw7-binding pocket can cause Notch pathway activation in T-ALL (Thompson et al., 2007). The intracellular forms of all four Notch proteins are potentially oncogenic and capable of transforming normal cells (Capobianco et al., 1997; Bellavia et al., 2000; Callahan et al., 2001; Kiaris et al., 2004).

Deregulated expression of Notch proteins, ligands, and targets has been described in a multitude of solid tumors, including cervical (Zagouras et al., 1995), head and neck (Leethanakul et al., 2000), endometrial (Suzuki et al., 2000), renal (Rae et al., 2000), lung (Dang et al., 2000), pancreatic (Miyamoto et al., 2003), ovarian (Hopfer et al., 2005), prostate (Santagata et al., 2004; Domingo-Domenech et al., 2012), ovarian (McAuliffe et al., 2012), esophageal (Subramaniam et al., 2012), oral (Liao et al., 2011), hepatocellular

(Wang, Xue, et al., 2009), and gastric (Yeh et al., 2009) carcinomas, osteosarcoma, mesothelioma (Bocchetta et al., 2003), melanoma (Balint et al., 2005), gliomas (Purow et al., 2005), medulloblastomas (Fan et al., 2004) and rhabdomyosarcoma (Raimondi et al., 2012). Dysregulation of Notch signaling has been reported in some hematological malignancies other than T-ALL. These include Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas (Jundt et al., 2002), some acute myeloid leukemias (AML) (Tohda et al., 2001), B-cell chronic lymphoid leukemias (B-CLL) (Hubmann et al., 2002; Hajdu et al., 2010) multiple myeloma (MM) (Houde et al., 2004; Jundt et al., 2004). For a recent review, see (Pancewicz et al., 2011).

Numerous studies have addressed the role of Notch in breast cancer. The first indication of a link between Notch signaling and breast cancer came from a study characterizing a frequent insertion site of the mouse mammary tumor virus (MMTV) in mice (Gallahan et al., 1987) which resulted in the overexpression of truncated Notch4 proteins. These truncated forms of Notch4 contained the transmembrane and intracellular domains, and similar to the truncated Notch1 subsequently discovered in T-ALL, they were constitutively active and caused spontaneous mammary tumors. This was confirmed when truncated Notch4, expressed in transgenic mice under the control of either the MMTV long terminal repeat or the whey acidic protein promoter (Jhappan et al., 1992; Gallahan et al., 1996) led to mammary carcinogenesis. Besides Notch4, there is evidence that constitutive activation of Notch1 and Notch3 in mouse models causes mammary tumors (Hu et al., 2006). Conversely, Notch2 has been associated with better prognosis in breast cancer (Parr et al., 2004).

Immunohistochemical studies reported that high level expression of Notch4 correlates with proliferative marker Ki67, while expression of Notch1 correlates with node status (Yao et al., 2011). Recent findings demonstrated that chromosomal rearrangements lead to the formation of Notch1 and Notch2 fusion transcripts in a subset of TNBC (Robinson et al., 2011). Fusion proteins behave as constitutively active Notch mutants. Tumors carrying such mutations may be sensitive to Notch inhibition. Recent data showing loss of function of Lfng suggest that it may be one of the most common genetic alteration resulting in hyperactive Notch signaling in breast cancer (Xu et al., 2012). Loss of expression of Lfng was seen in a majority of basal-like TNBC. In the same study, expression levels of Notch1 mRNA were shown to be highest in basal-like TNBC, followed by claudin-low TNBC. Expression levels of Notch1 mRNA are strongly correlated with poor survival in TNBC (Pallavi et al., 2012).

Mechanistically, Notch may contribute to carcinogenesis by inhibiting differentiation, inhibiting apoptosis or promoting proliferation. The intracellular forms of Notch induce transformation when it is expressed with oncoproteins that disable the G1-S checkpoint, such as adenovirus E1A, human papillomavirus E6 and E7, Ras, Myc, or SV40 large T-antigen. Depending on context, Notch also can activate the expression of several oncogenic pathways via direct or indirect induction of cyclins D1 (Cohen et al., 2010) and D3 (Joshi et al., 2009), cyclin A (Qi et al., 2003; Rizzo, Osipo, et al., 2008), SKP2 (Sarmiento et al., 2005), c-Myc (Liao et al., 2007; Hsu et al., 2008; Allen et al., 2011) or via activation of PI3K-AKT-mTOR, NF- κ B and NF- κ B2, β -catenin, signal transducers and activators of transcription-3 (STAT3). Notch can also co-operate with oncogenic pathways such as Wnt or Her2/Neu (reviewed in (D'Angelo et al., 2010)) as we describe above. Recent evidence suggests that Notch1 can induce expression of multidrug resistance transporter MRP1 (ABCC1) in breast cancer cells (Cho et al., 2011). Recent genetic studies in *Drosophila* indicate that transcription factor MEF (Myocyte-Specific Enhancer Factor, the *Drosophila* homologue of MEF2A, B, C, D in humans) potentiates the oncogenic activity of Notch^{IC}. In a broad range of human malignancies, expression of Notch1 and MEF2C were strongly correlated (Pallavi et al., 2012).

In addition to its cell-autonomous effects on oncogenic pathways, there is strong evidence for a role of Notch in tumor-stroma interactions. Notch signaling can mediate bidirectional tumor–stroma interactions and tumor– endothelium interactions (reviewed in (Gu et al., 2012)). For example, myeloma cells overexpress Jagged-2, activating Notch in stromal cells, which in turn produce IL-6, a growth factor for myeloma cells (Houde et al., 2004). Conversely, stromal cells express Jagged-1, activating Notch in myeloma cells (Jundt et al., 2004). Head and neck squamous cell carcinomas overexpress Jagged-1 which activates Notch in endothelial cells, promoting angiogenesis (Zeng et al., 2005). Productive tumor angiogenesis requires cooperation between VEGF and Notch signaling in the endothelium. Both DLL4 and Jagged-1 ligands participate in this process, with complementary roles (reviewed in (Gu et al., 2012)). Another poorly understood facet of the role of Notch in tumor microenvironment is the well-documented role of Notch signaling in a variety of immune system cells that can affect tumor growth through inflammation, angiogenesis and cytokines (reviewed in (Gu et al., 2012)).

In contrast to its oncogenic role in numerous tissues, Notch has a tumor suppressor effect in the epidermis. Notch1 induces differentiation in murine (Rangarajan et al., 2001) and human (Nickoloff et al., 2002) keratinocytes. This has been confirmed by tissue-specific ablation of Notch1 in conditional knockout mouse models (Nicolas et al., 2003). The mechanism for the tumor suppressor activity of Notch1 is still unclear. Cell-autonomous effects have been described, such as induction of p21 (Rangarajan et al., 2001), calcineurin (Mammucari et al., 2005) and IRF6 (Restivo et al., 2011). Additionally, Notch signaling is essential for epidermal differentiation / barrier formation as Notch1 KO skin loses barrier integrity leading to inflammation and production of cytokines such as TSLP-1 (Dumortier et al., 2010). Chronic inflammation and cytokine production in turn can lead to keratinocyte transformation, as well as systemic effects such as B-lymphocyte proliferative disorder (Demehri et al., 2008) or myeloproliferative syndrome (Dumortier et al., 2010). Recently, Notch1 inactivating mutations have been described in a subset of oropharyngeal squamous carcinomas, suggesting that Notch1 may have a direct or indirect tumor-suppressor role in some of these tumors (Agrawal et al., 2011; Elango et al., 2011). The role of the other 3 Notch paralogs was not investigated. Conversely, increased expression of Notch1 and Jagged-1 has been reported by other groups to be associated with poor prognostic characteristics in Asian head and neck squamous carcinomas (Zhang et al., 2009; Gu et al., 2010; Lin et al., 2010; Yu et al., 2012). This suggests molecular heterogeneity in these tumors. Whether this correlates with HPV status is currently unclear.

2.5 Notch signaling and cancer stem cells

In recent years, Notch activity has been reported in cancer stem-like cells (CSC) (reviewed in (Pannuti et al., 2010)). Notch activity has been implicated in the maintenance of this “cancer stem cell” phenotype in breast cancer (Farnie et al., 2007; Sansone et al., 2007; Harrison et al., 2010), embryonal brain tumors (Fan et al., 2006), glioma (Shih et al., 2006; Fan et al., 2011), hepatocellular carcinoma (Yao et al., 2009), pancreatic (Wang, Azmi, et al., 2009) and prostate carcinomas (Domingo-Domenech et al., 2012).

CSC are thought to constitute a small subset of cancer cells with stem-like phenotype that are a reservoir of self-sustaining cells with the ability to self-renew, presumably leading to recurrence. The stem-like phenotype is also characterized by enhanced resistance to chemo- and radio-therapy (reviewed in (Pannuti et al., 2010) and (Jang et al., 2012)). We demonstrated that breast CSCs of different subtypes and in secondary mammospheres from clinical specimens show higher levels of Notch activity compared with the majority of the tumor cells. Blockage of Notch by γ -secretase inhibitors (GSIs) impaired sphere formation, proliferation and anchorage independent growth in soft agar (Grudzien et al., 2010). This data supports a crucial role for Notch signaling in maintenance of breast cancer stem-like

cells and suggest that Notch inhibition may have clinical benefits in targeting them. Indeed, evidences showed in a Her2/Neu positive xenograft model (Pandya et al., 2011) indicate that GSIs used in combination with Herceptin do not increase the effects of Herceptin on tumor volume, but completely abrogate tumor recurrence. This strongly suggests an anti-CSC effect.

Thus, the structural differences observed between Notch proteins and its ligands, the ability of Notch receptors to interact with several types of ligands and regulatory or oncogenic proteins, the production of abnormal levels of Notch receptors or ligands offer a variety of potential targeting strategies, some non-selective and some selective for specific Notch receptors or ligands. The cross-talk with other pathways, which must be examined in specific indications, offers the potential for mechanism-based combinations.

3. Strategies to target the Notch signaling pathway

Based on our current understanding of the structure, function and regulation of Notch receptors and ligands, we can identify several steps that can potentially be targeted to inhibit Notch signaling: 1) expression of ligands, 2) ligand ubiquitination and trans-endocytosis, 3) expression of Notch receptors, 4) ligand-receptor binding, 5) heterodimer dissociation during Notch activation, 6) ADAM-mediated cleavage of Notch, 7) subsequent ubiquitination and endocytosis of the γ -secretase substrate, 8) γ -secretase-mediated cleavage of Notch, 9) assembly of the coactivator complex with Notch and CSL, 10) heterodimerization of Notch transcriptional complexes, 11) Notch post-translational modifications and 12) expression of Notch targets. In the next section we will describe currently available Notch inhibitors that target several of these steps and their current development status.

3.1 Neutralizing Notch antibodies

Blocking monoclonal antibodies (mAb) directed against Notch 1, 2 and 3 are under investigation. Two classes of blocking anti-Notch antibodies had been developed. One is directed to the extracellular negative regulator region (NRR) of Notch, blocking the conformational change that allows the ADAM protease cleavage (Aste-Amezaga et al., 2010). A second class consists of ligand-competitors directed against the EGF-repeat region of Notch receptors, blocking the ligand binding domain (LBD) (Aste-Amezaga et al., 2010). Both NRR- and LBD-Notch antibodies induce a strong and specific downregulation of Notch1 signaling, but LBD required higher antibody concentrations to exert the inhibitory effects (Aste-Amezaga et al., 2010). Interesting, Notch 1 NRR (NRR1) antibodies are also capable to bind and inhibit Notch1 carrying the “class I” NRR mutations (single amino acid substitutions or short insertions or deletions in the NRR domain of Notch 1 that cause increased Notch1 activity) in T-All cells (Aste-Amezaga et al., 2010). NRR-specific anti-Notch1 (NRR1), Notch 2 (NRR2) and Notch 3 (NRR3) antibodies that bind to the extracellular binding domain of Notch have been developed and they are in preclinical or in *in vitro* studies (Li et al., 2008; Aste-Amezaga et al., 2010; Wu et al., 2010). NRR1 also showed anti-angiogenic effects, inhibited blood circulation to the tumor and dramatically inhibited tumor growth (Li et al., 2008; Aste-Amezaga et al., 2010; Wu et al., 2010). Based on the success of in *in vitro* and preclinical studies using blocking Notch antibodies, a dose escalation Phase I clinical trial of OMP-59R5, a humanized mAb that blocks Notch 2 and Notch 3 signaling, has been opened in metastatic or relapsed solid tumor patients who have received prior treatment with standard chemotherapeutic drugs. Some mAbs specific for the negative regulatory region of Notch3 have been shown to inhibit ligand-induced Notch activation by stabilizing the auto-inhibited conformation of the receptor and preventing heterodimer dissociation (Li et al., 2008).

Blocking antibodies against Notch ligands are under development. Anti-Dll4 mAb (Ridgway et al., 2006) and soluble Dll4-Fc fusion proteins (Noguera-Troise et al., 2006; Scehnet et al., 2007) that bind Notch receptors and prevent their activation by endogenous Dll4 have been generated. These biologics inhibited Notch signaling in endothelial cells, caused disorganized angiogenesis and inhibited tumor growth (Ridgway et al., 2006). They are therefore being investigated as anticancer treatments (Thurston et al., 2007; Yan et al., 2007). Studies using the humanized anti-Dll4 mAb OMP-21M18 that blocks the interaction with Notch1 and Notch4, showed anti-tumor activity in patient-derived xenografts independent of any effect on angiogenesis (Reynolds et al., 2011).

Clinical trials using the OMP-21M18 antibody were designed for patients with solid tumors as colorectal cancer, pancreatic cancer, and small cell cancer. Currently, four active clinical trials using OMP-21M18 are ongoing using it as a single agent (NCT00744562) or in combination with chemotherapy (NCT01189968, NCT01189942, NCT01189929) in different solid tumors. The mAb approach has the advantage of potentially exquisite specificity, with the disadvantages of mAbs including limited biodistribution and prolonged half-life. Specificity may decrease toxicity in cases where a specific Notch signaling protein is pathogenetically involved. On the other hand, when multiple Notch paralogs are involved, such as the case of Lfng-negative breast cancer, targeting of individual receptors may not be the most effective approach.

Recently, a novel mAb against the extracellular domain of nicastrin, A5226A, has been generated. This antibody recognizes fully glycosylated mature nicastrin in the active γ -secretase complex on the cell surface, and inhibits γ -secretase activity by competing with substrate binding *in vitro*. The A5226A antibody abolished the γ -secretase activity-dependent growth of T-ALL cell lines and tumor growth of a T-ALL xenografts mouse model (Hayashi et al., 2012). A different nicastrin mAb has anti-CSC and therapeutic activity in breast cancer models (Lombardo et al., 2012). Nicastrin mAbs would ideally cause γ -secretase inhibition (and potentially pan-Notch inhibition) without the potential off-target effects of small molecules.

3.2 Decoys

Decoys are soluble forms of the extracellular domain of Notch receptors or Notch ligands. Soluble decoys compete with their endogenous cell surface-bound counterparts and abrogate Notch signaling due to the lack of a transmembrane region necessary for receptor activation. A Notch1 decoy that acts as a ligand-dependent Notch antagonist blocks Notch signaling in endothelial cells, affecting tumor neoangiogenesis and growth. It also reduces Notch1 activity and interferes with Dll1, Dll4 and Jagged1 activities, acting as a pan-ligand inhibitor (Funahashi et al., 2008). Soluble forms of the DSL type ligands Dll1 (Varnum-Finney et al., 2000) and Jagged1 (Small et al., 2001) have also been successfully used to inhibit Notch signaling. The presence of endogenous soluble Notch ligands has been reported as a result of endogenous metalloproteases activity (LaVoie et al., 2003; Six et al., 2003). Thus, there is evidence to support the use of soluble Notch ligands as a therapeutic tool. The extracellular domain of Dll1 can exist in a membrane-tethered and in a soluble form (Smas et al., 1997). Another non-canonical Notch ligand is EGF-like domain 7 (EGFL7), a secreted angiogenic factor expressed in endothelial cells. It binds the extracellular domain of the four Notch receptors and inhibits Notch activation induced by Jagged. EGFL7 inhibits neural stem cells renewal (Schmidt et al., 2009) and inhibits Notch activity in post-natal retina and in primary endothelial cells (Nichol et al., 2010). These results suggest that EGFL7 may be used as a Jagged antagonist in cancer cells. The potential efficacy of decoys will depend in large part on their pharmacokinetics and biodistribution. A decoy that achieves better biodistribution than mAbs inside solid tumors may be an attractive therapeutic candidate.

3.3 γ -secretase inhibitors (GSI)

The activation of Notch depends largely on γ -secretase activity (Aster et al., 2008). Thus, γ -secretase is a promising target for Notch inhibition. Non-selective GSIs, often referred to as “Notch inhibitors” in oncology are widely assumed to be equivalent in terms of biological activity and have cytostatic or cytotoxic activities in various cancer cells. GSIs are in clinical trials in a variety of indications (Tammam et al., 2009; Wei et al., 2010; Fouladi et al., 2011; Pandya et al., 2011). Several chemical classes of GSIs have been developed. Most of them are competitive inhibitors of the catalytic activity of presenilins. The dipeptide inhibitor, z-Ile-Leu-CHO (GSI-I) was showed to have Notch1-dependent anti-neoplastic activity in Ras-transformed fibroblasts (Weijzen et al., 2002) and induced apoptosis in melanoma xenografts (Qin et al., 2004). A similar tripeptide inhibited the proliferation of MDA-MB231 cells and tumor growth in MDA-MB231 xenografts. It also inhibited the growth of ER α ⁺ T47D:A18 cells and had a synergistic inhibitory effect with Tamoxifen on ER α ⁺ xenografts (Rizzo, Miao, et al., 2008). These peptides, however, are not candidate human drugs due to poor pharmacokinetics and off-target effects. GSI Compound E inhibited growth and induced apoptosis by increasing the G0/G1 fraction and decreasing the S-phase fraction in T-ALL cell lines (Weng et al., 2003). LY411,575, a GSI that binds to presenilin 1 (PS1) has been widely used in Alzheimer’s disease, where it reduced the accumulation of amyloid- β peptide (Wong et al., 2004; Hyde et al., 2006). In the HER2⁺ breast cancer cell line BT474, LY 411,575 treatment increased apoptosis and re-sensitized resistant HER2⁺ cells to trastuzumab (Osipo, Patel, et al., 2008). GSI MRK-003, the parent compound of clinical agent MK-0752, had good preclinical activity in breast cancer and T-ALL (Tammam et al., 2009; Pandya et al., 2011). This compound is more effective than LY411,575 in human mammospheres (Grudzien, 2010) and it completely abrogates recurrence in HER2⁺ xenografts (Pandya, 2011) in combination with trastuzumab. The clinical compound, MK-0752, also binds to PS1. It is currently in several Phase 1 clinical trial for pediatric and adult oncology treatment (Macy et al., 2008; Zweidler-McKay, 2008; Zhou et al., 2009; Fouladi et al., 2011). We have completed a pilot clinical trial with MK-0752 in combination with endocrine therapy in the preclinical setting (Albain, 2011). This combination was safe and well tolerated, and, importantly, showed molecular evidence of anti-proliferative and pro-apoptotic effects in tumor tissue. GSI RO4929097 appears to differ from other GSIs in that it induces a “less transformed” and slower-growing tumor phenotype without appreciable pro-apoptotic effects (Luistro et al., 2009). Whether this is due to selectivity for specific Notch paralogs is unclear. Currently it is being evaluated in several NCI-sponsored phase 1 clinical trials for treatment of solid tumors and T-ALL. We (Means-Powell JA, 2012) have just completed a phase 1b trial of this agent in combination with exemestane in metastatic, ER⁺ breast cancer. The combination was well-tolerated and clinical response was observed in a significant number of patients. Unfortunately, the development of RO4929097 has been hampered by its pharmacokinetic liability, due to auto-induction of hepatic metabolism. GSI PF-03084014 has shown an effect in tumor growth and inducing apoptosis in several tumors (Wei et al., 2010) and it is currently in Phase 1 clinical trials for T-All and solid tumors. BMS-708163 is a Notch-selective, second-generation GSI which is in phase 1 (NCT01454115, NCT00860275, NCT01039194, NCT01002079, NCT01079819). *In vivo*, most GSIs show evidence of anti-angiogenic effects in addition to direct effects on tumor cells. This is most likely due to inhibition of the Notch-VEGF cross-talk essential for angiogenesis (see above). The relative importance of anti-angiogenic versus direct anti-tumor effects in the *in vivo* mechanism of action of GSIs is still unclear, and may depend on tumor model and class of GSIs. Interestingly, pro-angiogenic cytokines IL6 and IL8 have been reported to cause resistance to GSI RO4929097 (He et al., 2011). The possible role of Notch inhibition in other tumor-stroma components, including T-cells, macrophages, tumor-associated fibroblasts and others is poorly understood.

In summary, it is safe to say that GSIs have shown anti-tumor effects in numerous preclinical models. Anti-angiogenesis and anti-CSC effects are likely to contribute to their mechanism of action *in vivo*. Due to the broad spectrum of substrates of γ -secretase, GSIs are likely to have multiple off-target effects *in vivo*. Their toxicity, however, appears to be almost exclusively Notch-mediated. The most serious adverse effect is diarrhea, caused by goblet cell metaplasia of the small intestine which in turn is due to Notch inhibition in intestinal epithelial stem cells. This effect can be dose-limiting and in many cases it requires intermittent administration. The relative lack of specificity of GSIs is not necessarily a therapeutic problem, and may even be an advantage provided that mechanistically relevant pharmacodynamic biomarkers are identified. However, successful development of these agents will require evidence of target inhibition in tumor tissue to guide dose escalation. Molecular biomarkers indicative of Notch inhibition may differ in different tumors and the classical Notch targets (e.g., HES1) may not be the best biomarkers. In our experience, genes responsive to Notch in ER+ breast cancer cell lines have been confirmed to be modulated by GSIs in patient tissue (Means-Powell JA, 2012) more reliably than “classical” Notch target genes described in the literature. Whenever possible, neo-adjuvant clinical trials guided by strong preclinical evidence may be the best approach to development.

3.4 Blocking peptides

Numerous studies on Notch signaling have demonstrated that the activation of Notch and its nuclear access are required to maintain tumor cell growth and survival. Thus, blocking the transcriptional nuclear complex formed by Notch, CSL and coactivators may be another possible therapeutic tool. In 2003, the first dominant negative peptide derived from MAML1 was described. This peptide forms a transcriptionally inert complex with Notch1 and CSL. It has been shown to inhibit the growth of transformed T-ALL cell lines (Weng et al., 2003). Six years later, a new synthetic, cell-permeable, stabilized α -helical, hydrocarbon-stapled peptide derived from MAML1 was generated (SAHM1) (Moellering et al., 2009). Stapled peptides are a new generation of drugs consisting in peptides outfitted with chemical braces or “staples” (Moellering et al., 2009). SAHM1 peptide showed a direct binding to pre-assembled Notch1–CSL complexes and competitive inhibition of the MAML1 co-activator binding. In addition, SAHM1 induced a direct transcriptional repression that resulted in anti-proliferative effects on T-ALL cell lines. SAHM1 treatment also showed an inhibition of leukemic progression through inhibition of Notch signaling in a murine model of T-ALL (Moellering et al., 2009).

The use of stabilized, cell-permeable peptides to interfere with protein complex formation possesses several attractive features; these molecules have relatively small size, they have a high structural compatibility with target proteins, and have the ability to disrupt protein-protein interfaces. Pharmacokinetics will dictate to what extent these molecules can be used therapeutically in humans. Another consideration is the relative role of canonical (nuclear) versus non-canonical Notch signaling. In situations where non-canonical Notch signaling may be responsible for oncogenic activity, such as with Notch4 in the mammary gland (Raafat et al., 2009), inhibition of canonical Notch–CSL-mediated transcription may not be ideal. Having said that a MAML-mimetic may sequester N^{IC} in inactive complexes with CSL, potentially reducing the overall cellular concentration of N^{IC} and also inhibiting non-nuclear Notch signaling.

3.5 Natural compounds

Natural dietary supplements have received much attention, primarily because epidemiological studies have shown that the consumption of fruits, soybean and vegetables is associated with reduced risk of several types of cancers (Lee et al., 2003; Smith-Warner et al., 2003). Such compounds or mixtures of compounds have notoriously pleiotropic

activities, but in many cases their biological effects are very promising. As a result, many groups have focused on elucidating molecular mechanisms and identifying targets of these natural products. Several dietary derived compounds target Notch signaling. Isoflavone genistein, found in soy products, inhibits Notch signaling, decreases cell proliferation and induces apoptosis in pancreatic cancer cells via downregulation of NF- κ B activity (Wang et al., 2006a). In prostate cancer cells, genistein reduces cell viability and induces apoptosis through downregulation of Notch1, AKT and FoxM1 (Wang, Li, et al., 2011). Sulforaphane, a natural compound derived from cruciferous vegetables such as broccoli, inhibits breast CSCs growth *in vitro* and *in vivo* through down-regulation of the Notch and Wnt/beta-catenin pathways, and inhibits growth of CSC-xenografts derived from prostate and pancreatic tumors (Kallifatidis et al., 2011). Quercetin is a major polyphenol and flavonoid commonly found in many fruits and vegetables. It has been reported that quercetin decreases the levels of Notch1 protein and its active fragment in a leukemia cell line with constitutive Notch1 activation (Kawahara et al., 2009) and has a synergistic effect with GSIs on Notch1 activity (Okuhashi et al., 2011). Quercetin also targets CSCs and the epithelial-mesenchymal transition (EMT) phenotype of pancreatic cancer cells (Zhou et al., 2010). Curcumin is an active compound found in *Curcuma longa*, which is widely used as a flavoring agent in food (e.g., turmeric). It has been shown to have antitumor activity. Curcumin downregulates Notch1 and induces apoptosis through inactivation of NF- κ B in pancreatic cancer cells (Wang et al., 2006b) and in oral cancer cells (Liao et al., 2011). Resveratrol, a polyphenolic compound found in grapes, red wine, purple grape juice, peanuts, and some berries, induces apoptosis in part by inhibiting Notch and PI3K/AKT in T-ALL cells (Cecchinato et al., 2007) and in glioblastoma cells (Lin et al., 2011). Recently, it has been reported that resveratrol can also activate Notch2 as a mechanism of apoptosis induction in medullary thyroid cancer (Truong et al., 2011) and in carcinoid (Pinchot et al., 2011). Notch2 may function as a Notch1 antagonist due to its lower transcriptional activity.

Considering the relatively low toxicity of natural products, the idea of such compounds inhibiting Notch in tumor cells or in CSC is potentially attractive. Chronic, partial Notch inhibition by natural products may contribute to chemopreventive activity. Therapeutic uses in established cancers are likely to require combinations with conventional chemotherapeutic agents.

4. Conclusions

In this brief commentary, we attempted to summarize the role of Notch proteins in cancer and current knowledge on Notch-targeting therapeutic tools. Deregulation of Notch proteins has been associated with specific pathologies including cancer development and progression, and with the self-propagation of cancer stem cells. These and other features of Notch signaling, identify Notch as a candidate diagnostic and prognostic biomarker, and an attractive target for cancer therapy. Currently, most Notch-directed therapies involve the use of GSIs, but a variety of biopharmaceuticals and natural products deserve further investigation. As is the case for most embryonic/CSC pathway inhibitors, the development of Notch inhibitors will need to be guided by biology. Biomarkers indicative of Notch activity (and of its inhibition by investigational drugs) will have to be identified and validated in each indication. Additionally, mechanism-based combinations will play a key role. We have demonstrated that combinations with endocrine therapy and trastuzumab can have remarkable therapeutic activity in breast cancer models compared to single agent treatment. Importantly, in the case of Her2-positive breast cancer the effect of Notch inhibition was to prevent recurrence rather than to decrease tumor volume (Pandya et al., 2011). This implies that tumor volume may not be the most informative endpoint in clinical trials of Notch-targeting agents. Recurrence-free survival and/or good surrogate endpoints predictive of survival (e.g., circulating tumor cells, mammosphere-forming cells) are likely

to be more informative. These challenges do not diminish the tremendous therapeutic opportunity offered by a pathway that is essential for CSC maintenance, angiogenesis and in many cases proliferation and survival of cancer cells.

Acknowledgments

This work was financially support by NIH PO1AG025531. We thank Dr. Christian R. Gomez for his critical review and comments on the manuscript.

Abbreviations

ADAM10/17	A Disintegrin And Metalloprotease 10/17
AIP4	Atrophin-1 interacting protein 4
Akt	Protein Kinase B
ANK	Ankyrin repeats
AP2	Adaptor protein 2
APH1	Anterior Pharynx-defective 1
B-CLL	B-cell chronic lymphoid leukemias
bHLH	basic helix-loop-helix
Ca²⁺	Calcium
Cbl	Casitas B-lineage lymphoma
CBP/p300	CREB binding protein (CBP) and p300
CDK	Cyclin-dependent kinase
ChIP-Seq	chromatin immunoprecipitation and sequencing
CSC	<u>C</u> ancer <u>S</u> tem <u>C</u> ells
CSL	CBF-1, Suppressor of Hairless/LAG1
Csf	Granulocyte colony stimulating factor
CIR	CSL Interacting Repressor
DLL 1, 3, 4	Delta-like-1, -3, -4
DLK1/2	delta-like 1/2 homolog (Drosophila)
DNER	Delta and Notch-like epidermal growth factor repeat
DSL	Delta/Serrate/Lag-2
EBF	Early B cell factor
EBNA2	Epstein-Barr virus nuclear antigen 2
EGF	<u>E</u> pidermal <u>G</u> rowth <u>F</u> actor
EGFL7	EGF-like domain-containing protein 7
ERK	Extracellular signal-regulated kinases
ERα	Estrogen receptor alpha
ETS	E-twenty six
Fbw7	F-box and WD40 repeat domain-containing 7

GCN5	General control of amino acid synthesis protein 5
GSK3β	<u>G</u> lycogen <u>S</u> ynthase <u>K</u> inase 3 beta
GSI s	<u>G</u> amma <u>S</u> ecretase <u>I</u> nhibitors
HD	Heterodimerization domain
HDAC	Histone deacetylase 1
HER2	Human Epidermal Growth Factor Receptor 2
HES1–5	<u>H</u> airy/ <u>E</u> nhancer of <u>S</u> plit family 1
HRT	Hairy-related family
IKKα	IkappaB kinase alpha
IRF6	Interferon regulatory factor 6
Lfng	Lunatic fringe
LSD1	Lysine-specific demethylase 1
LBD	Ligand binding domain
LN	Cystein rich-LIN12/Notch repeats
mAbs	monoclonal antibodies
MAML1	Mastermind-like 1
MEF	Myocyte Specific Enhancer Factor
Mfng	Manic fringe
MLK	Mixed lineage kinases
MMTV	Mouse mammary tumor virus
N^{IC}	Notch intracellular domain
N^{EC}	Notch extracellular domain
NTM	Notch transmembrane domain
N-CoR	Nuclear receptor co-Represor
NEDD4	<u>N</u> eural precursor cell expressed <u>D</u> evelopmentally <u>D</u> own-regulated 4
NEXT	Notch Extracellular Truncation
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NRR	Negative regulatory region
LCLs	Lymphoblastoids cells
LN	Cysteine-rich Lin12/Notch repeats
OFUT1	O-Fucosyltransferase 1
OPA	Polyglutamine stretch
PCAF	P300/CBP-associated factor
PHF8	PHD finger protein 8
PEN2	Presenilin Enhancer 2

PEST	Proline, Glutamic acid, Serine and Threonine
PDZL	Post synaptic density protein (PSD95)/Drosophila disc large tumor suppressor (Dlg1)/Zonula occludens-1 protein (zo-1) Ligand
PEST	Proline, Glutamic acid, Serine and Threonine
PS1	Presenilin 1
PPAR	Peroxisome proliferators-activated receptors
RAM	RBP-jk association module
RBP-jk	Recombining binding protein suppressor of hairless
Rfng	Radical fringe
RUNX	Runt-related transcription factor
SHARP	SMRT/HDAC-1-associated repressor protein
SKP2	S-phase kinase-associated protein 2
SMRT	Silencing Mediator for Retinoid and Thyroid receptor
STAT3	Signal transducer and activator of transcription 3
T-ALL	T-cell Acute Lymphoblastic Leukemia
TAD	Transactivation domain
TCRβ	T-cell receptor beta
TGFα	Transforming growth factor alpha
TNBC	Triple negative breast cancer
TSLP-1	thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
ZNF143	zinc finger protein 143

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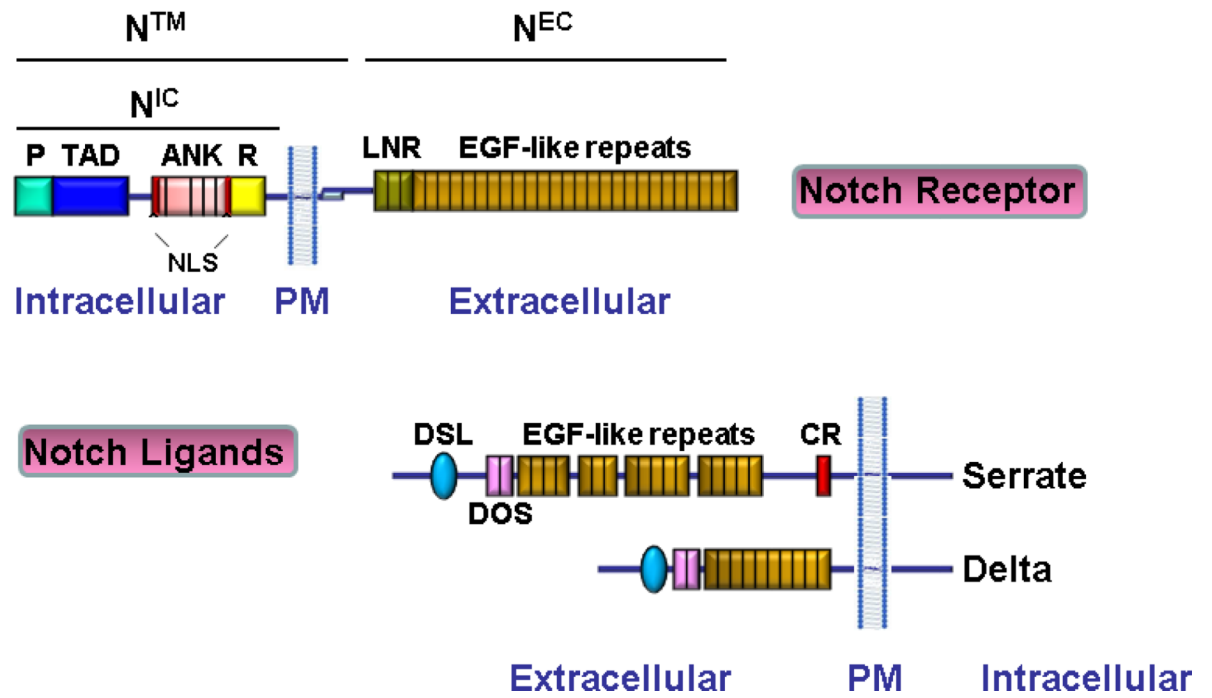
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A. Drosophila



B. Mammals

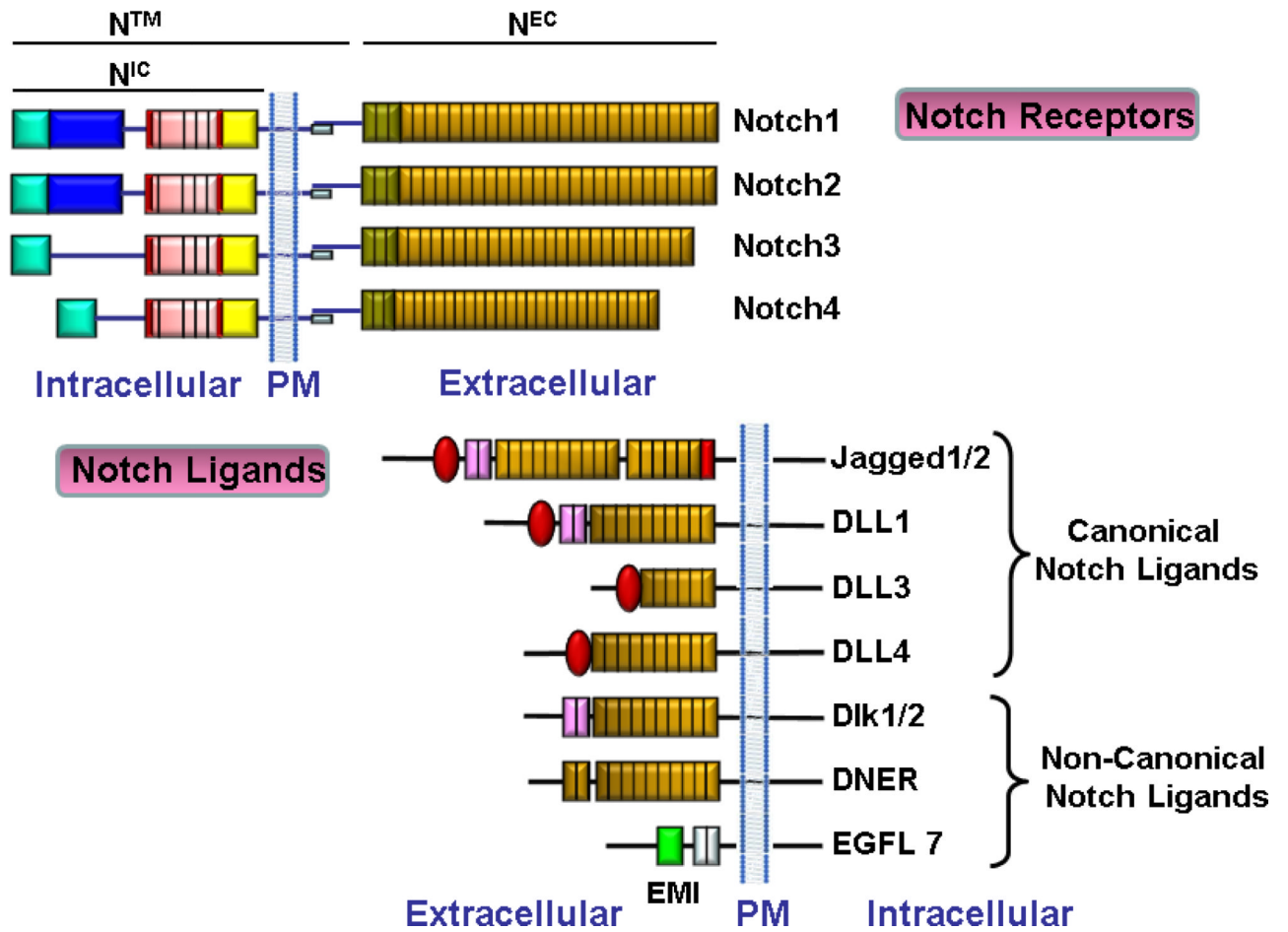


Fig. 1.
Notch receptors and ligands

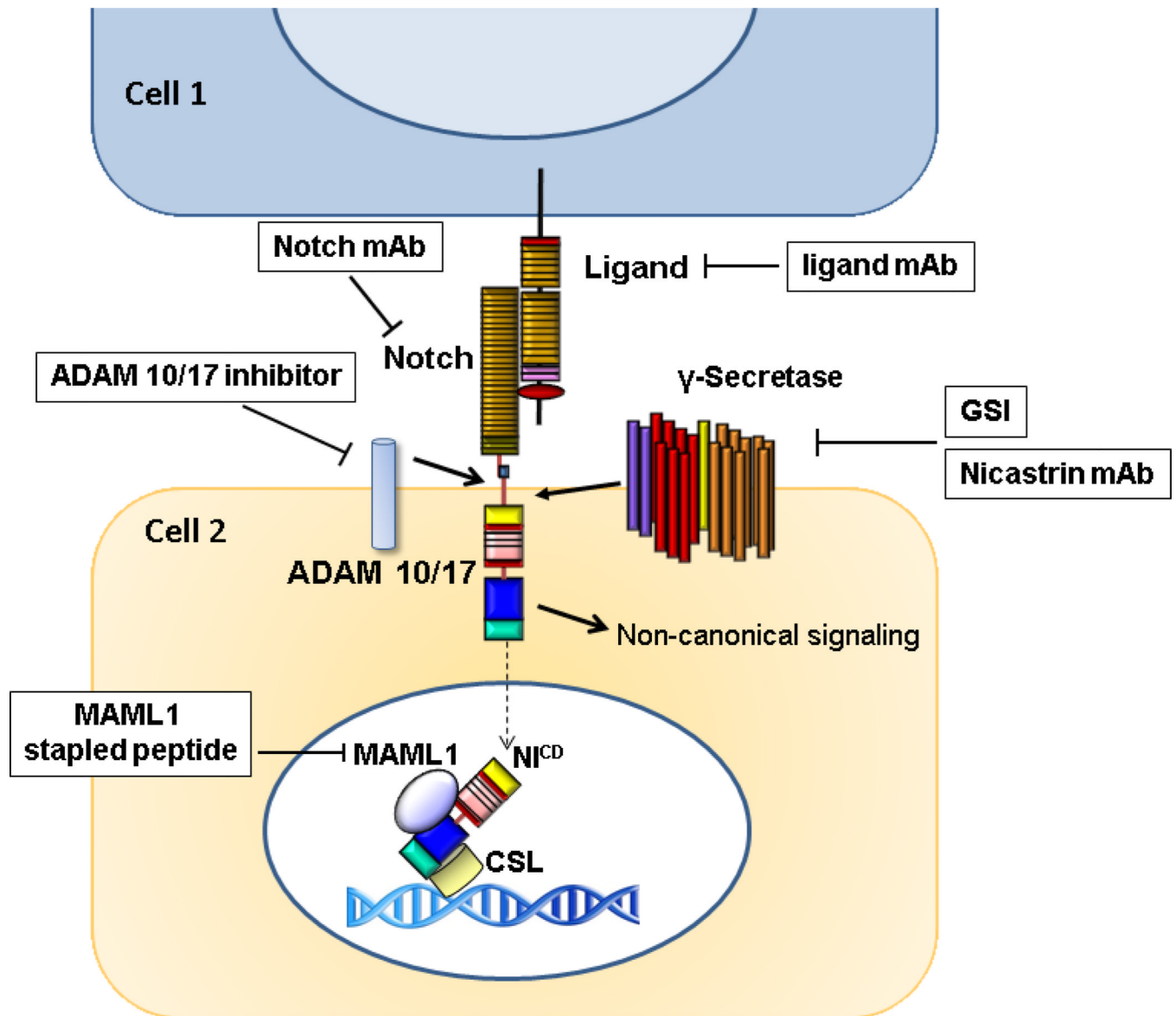


Fig 2.
Notch signaling pathway

Table I

Notch inhibitors and their current development stage

Agent	Notch pathway target	Compound	Condition	Development Phase
Neutralizing antibodies	Interference with ligand-induced Notch subunit separation and Notch ligands. Specific for Notch 1, 2, 3; DLL1, 4	OMP-59R5 anti-Notch2/3 mAb (OncoMed Pharmaceuticals)	Solid tumors	Phase 1 NCT01277146
		NRR1 anti-Notch1 mAb (Genentech and Exelixis; Merck)	Breast cancer Colon Cancer Anaplastic carcinoma T-cell leukemia T-ALL cell line (Li et al., 2008; Aste-Amezaga et al., 2010; Wu et al., 2010)	Preclinical and <i>In vitro</i> studies
		NRR2 anti-Notch2 mAb (Genentech and Exelixis)	Breast cancer Colon Cancer Anaplastic carcinoma HEK293T cell line (Wu et al., 2010)	Preclinical studies
		NRR3 anti-Notch3 mAb (Genentech)	HEK293T cell line (Li et al., 2008)	<i>In vitro</i> studies
		OMP-21M18 anti-DLL4 mAb (OncoMed Pharmaceuticals)	Colorectal cancer Small cell lung cancer Pancreatic cancer Solid tumors	Phase 1 NCT01189929 NCT01189942 NCT01189968 NCT00744562
		DLL1-Fc and JAG1-Fc Anti-Delta-like1 and Jagged 1 Fc chimeric mAbs	Autoimmune encephalomyelitis (Fischer et al., 2011; Reynolds et al., 2011)	<i>In vitro</i> studies
		A5622A Anti-nicastroin mAb	T-cell leukemia T-ALL tumor (Hayashi et al., 2012)	T-cell leukemia T-ALL tumor (Hayashi, I, 2011)
Decoys	Interference with ligand-receptor interaction	Soluble forms of Notch1, Dll1 and Jagged 1	Endothelial cells (Varnum-Finney et al., 2000; Small et al., 2001; Funahashi et al., 2008)	Preclinical studies
γ-Secretase Inhibitor (GSI)	Notch 1, 2, 3, 4; Notch ligands	RO4929097 (Roche)	Breast cancer Brain tumors Colorectal cancer Melanoma Solid tumors T-cell leukemia	Phase 1 NCT01088763 NCT01198535 NCT01149356 NCT01141569 NCT01196416 NCT01218620 NCT01217411 NCT01270438 NCT01238133 NCT01208441
		MRK-003 (Merck)	Breast cancer T-cell leukemia (Tammam et al., 2009; Pandya et al., 2011)	Preclinical studies

Agent	Notch pathway target	Compound	Condition	Development Phase
		MRK-0752 (Merck)	Breast cancer Brain tumors Neoplasms Pancreatic cancer T-cell leukemia	Phase 1 NCT00756717 NCT00803894 NCT01295632 NCT01098344 NCT00645333 NCT01243762 NCT00572182 NCT00106145 NCT00100152
		PF-03084014 (Pfizer)	Neoplasms Solid tumors Lymphoid leukemia T-cell leukemia	Phase 1 NCT00878189
		MRK-0752 (Merck)	Breast cancer Brain tumors Neoplasms Pancreatic cancer T-cell leukemia	Phase 1 NCT00756717 NCT00803894 NCT01295632 NCT01098344 NCT00645333 NCT01243762 NCT00572182 NCT00106145 NCT00100152
		PF-03084014 (Pfizer)	Neoplasms Solid tumors Lymphoid leukemia T-cell leukemia	Phase 1 NCT00878189
Blocking peptide	Interference with Notch nuclear co-activator MAML1	MAM peptide antagonist SAHM1 (Aileron Therapeutics)	T-cell leukemia T-ALL tumors (Weng et al., 2003; Moellering et al., 2009)	Preclinical studies
Natural compounds	Downregulation of Notch activity and Notch pathway	Genistein Sulforaphane Quercetin Curcumin Resveratrol	Pancreatic cancer Prostate cancer Thyroid cancer Carcinoid T-ALL cells Glioblastoma cells Oral cancer cells (Wang et al., 2006; Cecchinato et al., 2007; Kawahara et al., 2009; Kallifatidis et al., 2011; Liao et al., 2011; Okuhashi et al., 2011; Pinchot et al., 2011; Truong et al., 2011; Wang et al., 2011)	Preclinical and <i>In vitro</i> studies