Drugs of the Future 2011, 36(9): 677-689

CCC: 0377-8282/2011
DOI: 10.1358/dof.2011.36.9.1678340

REVIEW ARTICLE

γ-SECRETASE INHIBITORS IN SOLID TUMOR MALIGNANCIES

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SUMMARY

The Notch pathway, like other developmental pathways such as hedgehog and Wnt, has diverse pleiotropic functions. The Notch pathway has a well-recognized role in hematological malignancies and recent research has also established a similar role in multiple solid tumors. This pathway has since received widespread attention as a promising target for cancer therapy. Here we provide a comprehensive overview of the pathway and review the role of the Notch pathway in the initiation, maintenance and progression of cancer. We further discuss the role of inhibitors of γ-secretase, a key enzyme of this pathway, in cancer therapy, with an emphasis on ongoing and recently completed trials assessing these agents in multiple tumor types.

INTRODUCTION

Notch is an evolutionarily conserved pathway that is important in embryonic development and for maintaining proper organ function in adult tissues by influencing cell fate decisions. The canonical Notch pathway inhibits the differentiation of stem/precursor cells by signaling through the Notch intracellular domain (NICD) and mastermind complex. Activation of the canonical Notch receptors involves the ligands Delta-like proteins 1 (Delta1, DLL1), 3 (Delta3, DLL3) and 4 (Delta4, DLL4) and protein jagged-1 (jagged1) and -2 (jagged2). The noncanonical Notch pathway induces the differentiation of progenitor cells via signaling through the NICD and protein Deltex complex. In contrast to the canonical Notch ligands Delta-like proteins and jagged, the noncanonical Notch pathway is activated by other ligands, such as contactin-1/neural cell surface protein F3 (1). Once cleaved, NICD has been shown to interact with non-CSL targets, including β-catenin (2) and hypoxia-inducible factor 1-α (HIF-1-α) (3). In addition, there is accumulating evidence that NICD functions as a scaffold promoting the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (4). Therefore, noncanonical Notch mediates a subset of cellular effects independent of the typical responses induced by jagged and Delta-like proteins. Dysregulation of the Notch signaling pathway has been implicated in tumorigenesis. In this review, we focus on the canonical Notch pathway and examine: 1) the components and activation of Notch signaling; 2) the role of Notch in tumorigenesis; and 3) γ-secretase inhibitors in early-phase clinical trials.

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COMPONENTS OF THE NOTCH SIGNALING PATHWAY

There are many different components of the Notch signaling pathway. There are four Notch receptors (Notch 1-4) and five ligands (Delta1, Delta3, Delta4, jagged1 and jagged2) (Fig. 1A). The interaction between ligand and receptor among adjacent cells results in proteolytic cleavage by the γ-secretase complex of the Notch receptor and subsequent translocation of the NICD, which leads to the association with a transcriptional complex and transcription of Notch target genes (Fig. 1B).

Notch receptors

In mammals, the Notch receptors 1-4 are type I transmembrane proteins that are synthesized in the endoplasmic reticulum and cleaved in the Golgi apparatus, yielding a covalently linked heterodimeric protein that is expressed on the cell surface (5). The extracellular domain of the receptor contains epidermal growth factor (EGF)-like tandem sequences that range from 29 to 36 repeats. Notch 1 and 2 have 36 repeats, whereas Notch 3 and Notch 4 have 34 and 29 repeats, respectively. These specific regions are responsible for the binding of calcium ions altering the structure of the receptor, as well as interacting with ligands (6, 7). In particular, repeats 11-12 are important for binding ligands on neighboring cells (8). Following the EGF repeats, there is a negative regulatory region consisting of three cysteine-rich Lin-12/Notch repeats (LNRs) that are essential for

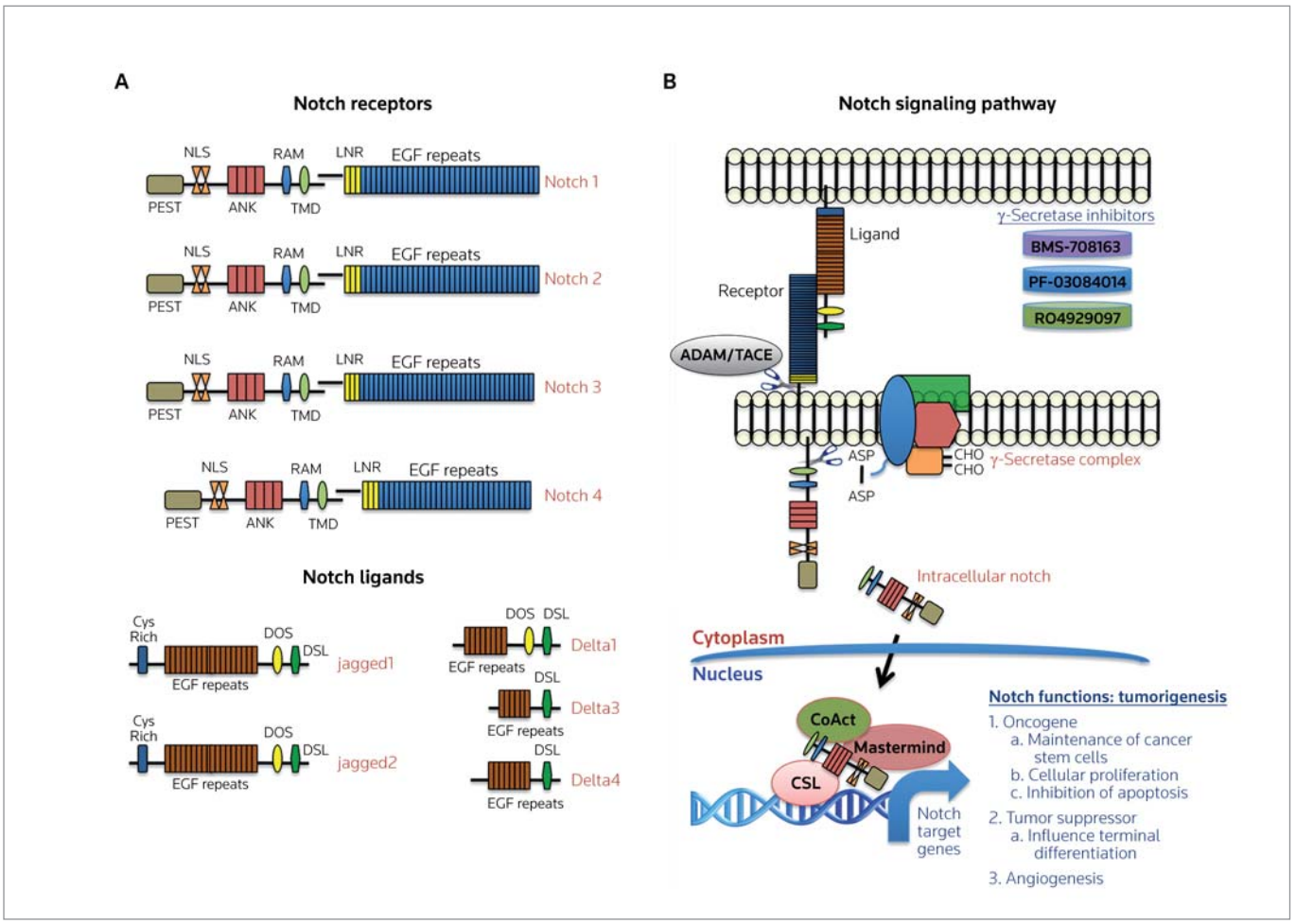


Figure 1. Notch receptors, ligands and activation of the Notch signaling pathway. (**A**) The Notch receptors (1-4) consist of epidermal growth factor (EGF)-like tandem repeats that are important for binding ligands, the negative regulatory region Lin-12/Notch repeats (LNR) and a transmembrane domain (TMD) recognized and cleaved by the disintegrin and metalloproteinase domain-containing protein 17 (ADAM 17)/TNF-α-converting enzyme (TACE) complex. Intracellular components of the notch receptor include an RBP-Jκ association module (RAM) and an ankyrin (ANK) domain that bind the transcription factor CBF-1/RBP-Jκ/Su(H)/Lag-1 (CSL), two nuclear localization sequences (NLS) and proline/glutamic acid/serine/threonine-rich (PEST) motifs that enhance NICD stability. The ligands harbor EGF-like repeats that are important for interaction with the receptor. A cysteine-rich domain is present on jagged and absent on Delta ligands. Jagged1, jagged2, Delta1 have a Delta and OSM-11-like protein domain (DOS), while Delta3 and Delta4 lack this domain. **(B)** Interaction between Notch ligand and receptor among adjacent cells results in the proteolytic cleavage of the extracellular portion of the Notch receptor by ADAM/TACE. This is followed by the cleavage of the Notch intracellular domain by the γ-secretase complex and subsequent translocation of the intracellular domain (ICD) into the nucleus, where it associates with a transcriptional complex (CSL and mastermind), which culminates in the transcription of Notch target genes. The γ-secretase inhibitors MK-0752, PF-03084014 and RO4929097 are currently being evaluated in early-phase clinical trials.

inhibiting the pathway in the absence of ligands and a transmembrane domain (TMD). Point mutations within the TMD of Notch 1 have been identified in patients with T-cell acute lymphocytic leukemia (ALL) (9). The intracellular portion of the receptor harbors a recombining binding protein suppressor of hairless (RBP-Jκ) association module (RAM) and an ankyrin (ANK) domain, which are important for binding the transcription factor CBF-1/RBP-Jκ/Su(H)/Lag-1 (CSL) in the nucleus after receptor cleavage (10-12), two nuclear localization sequences (NLS), and proline/glutamic acid/serine/threonine-rich (PEST) motifs that enhance NICD stability (13). A transactivating domain has been described in Notch 1 and 2, while Notch 3 and 4 lack this domain (14). Whether these differences in the extracellular and/or intracellular regions constitute diversity in biological responses remains to be elucidated.

Notch ligands

In mammals, the Notch ligands Delta1, Delta3, Delta4 and jagged1 and jagged2 are type I transmembrane proteins. Similar to the Notch receptors, the ligands have EGF-like repeats that vary in num-

ber and have the potential for binding calcium ions (15). Delta and jagged ligands contain an *N*-terminal DSL motif (Delta/Serrate / Lag-2) that, in conjunction with EGF repeats, is important for interaction and activation of the Notch receptor (16). Jagged ligands contain a cysteine-rich domain within the DSL motif, whereas cysteine-rich domains are absent in Delta. Some of the ligands, such as jagged1, jagged2 and Delta1, have a Delta and OSM-11-like protein domain (DOS), whereas Delta3 and Delta4 lack this domain; however, it has been demonstrated that Delta3 and Delta4 have the potential to interact with DOS co-ligands (13). DOS domains have also been shown to enhance binding to Notch receptors. Interestingly, Notch ligands have been shown to thwart activation of the Notch receptor through *cis* interactions on the same cell (17, 18).

Activation of the receptor

A unique feature of the Notch signaling pathway is that it does not require the assembly and activation of secondary messengers at the intracellular receptor complex that subsequently transmits downstream signals; instead, this pathway is activated through a sequence of proteolytic events. The binding of the ligand to the receptor leads to the exposure of the cleavage site of approximately 12 amino acids (S2) before the TMD (19). The S2 site is recognized and cleaved by the disintegrin and metalloproteinase domain-containing protein 17 (ADAM 17)/TNF-α-converting enzyme (TACE) complex, leading to the shedding of the Notch extracellular domain, producing an intermediate fragment known as Notch extracellular truncation (NEXT) (20, 21). This is followed by cleavage of the TMD within NEXT by the γ-secretase complex at sites 3 and 4, which ultimately releases NICD from the membrane (19, 22). The γ-secretase complex consists of four different proteins: presenilin, nicastrin, gamma-secretase subunit APH-1 (anterior pharynx defective 1) and gamma-secretase subunit PEN-2 (presenilin enhancer protein 2) (23-25). The aspartyl residues of presenilin are required for proteolytic activity, whereas APH-1 (assembly of the complex), nicastrin and PEN-2 are important for stabilizing the complex (26-30). The release of NICD from the membrane by the γ-secretase complex leads to the translocation of NICD into the nucleus, where a transcriptional complex forms through the interaction of NICD RAM and ANK domains with CSL (31). This interaction facilitates the recruitment of the co-activator mastermind to the complex, culminating in the upregulation of Notch target genes, such as *HES1*, *CDKN1A*, *HEY1* and *HEY2* (32-38).

EFFECTS OF THE NOTCH PATHWAY ON TUMOR BEHAVIOR

Normal function of the Notch pathway

The Notch signaling pathway influences many processes that are essential for proper development of the embryo. In particular, Notch signaling is required for the commitment of progeny to form all three germ layers (endoderm, ectoderm and mesoderm) during embryonic development (39-44). In addition, Notch is required for conservation and differentiation of tissue-specific stem cells that are vital for proper maintenance and function of organs. These pleiotropic effects are dependent on the context of the specific tissue and the signals the cell receives from the microenvironment.

There are several different models that explain the role of Notch in cell fate decisions within tissues. First, Notch activation preserves the stem cell population by preventing cellular differentiation (45). Blockade of the Notch pathway induces differentiation of proliferative cells in the intestinal crypts into goblet cells (46). Additionally, it has been shown that Notch inhibition results in an increase in neurogenesis, resulting in a diminished progenitor pool (47, 48). Another function of Notch pathway activation is influencing cell fate directionality (45). One suggested mechanism is lateral signaling, whereby cells that express both ligands and receptors eventually lose either the ligand or the receptor (49-52). As a result, only the cell with the Notch receptor will have activation of the pathway and retain stem cell properties, whereas the cell without the receptor will differentiate. An alternative mechanism has been proposed that involves the Notch pathway inhibitor protein numb homolog (Numb). During cell division, a mother cell accumulates Numb at one side of the cell, resulting in one daughter cell with the Notch pathway inhibitor Numb and the other daughter with activation of the pathway, altering the fate of these cells (53). This asymmetric division produces a differentiated progeny that is more specialized, while retaining the stem cell population. A third function of Notch is to enhance cell cycle progression of two developmentally similar cells to terminally differentiate (45). Since this pathway plays an important role in influencing many cellular processes, including stem cell maintenance, cellular proliferation, differentiation and apoptosis, dysregulation of this pathway has been implicated as a contributor to malignant transformation of cells in many solid tumors.

Effects of Notch on tumorigenesis

Elevated expression of Notch receptors, ligands and target genes has been observed in many different solid tumors, including breast, lung, cervical, colon, pancreas, skin and brain tumors (54-61). It is believed that Notch is oncogenic in tissues where its main function is to sustain the stem cell population and influence binary cell fate decisions, and tumor suppressive in cells where it promotes terminal differentiation (62). The oncogenic role of the Notch pathway influences many different genes that are involved in cellular proliferation and in inhibiting apoptosis. One way Notch promotes growth is through the transcriptional activation of transcription factor HES-1 and NF-κB 2. HES-1 has been shown to decrease the cyclin-dependent kinase inhibitor $p27^{kip1}$ (63), whereas NF-κB 2 regulates many genes involved in enhancing cellular survival (64). Activation of the PI3K pathway by Notch has also been described, although the mechanism is not well understood (65). Despite the involvement of the Notch pathway in enhancing cellular survival, Notch alone is incapable of inducing malignant transformation of cells. For example, it has been demonstrated in different cell types that transfection of NICD itself was not sufficient to induce transformation, but when coupled with human papillomavirus (HPV) E6 and E7 or the oncogene *KRAS* transformation occurred (65, 66). Additionally, in breast cancer cell lines Mittal et al. (67) showed that Notch in conjunction with oncogenic *KRAS* was required for transformation.

In contrast to promoting cellular proliferation, Notch activation has been identified as tumor suppressive in carcinomas of the skin. In particular, Notch has been demonstrated to contribute to terminal differentiation of proliferating kerinatocytes by enhancing cell cycle arrest (34). Loss of Notch in the epidermis is accompanied by hyperplasia, which ultimately results in spontaneous basal cell carcino-

mas (68). Additionally, Nicolas et al. (69) investigated the loss of skin-specific Notch 1 in mice to evaluate the long-term effects of Notch deficiency. Mice lacking Notch 1 had an increase in hyperplasia of the epidermis that eventually resulted in basal cell carcinoma-like tumors. Evaluation of primary keratinocytes in the skin showed elevated levels of the hedgehog pathway gene *GLI2,* suggesting that Notch plays a role in inhibiting the hedgehog pathway, consequently leading to terminal differentiation of keratinocytes.

Role of Notch in maintenance of cancer stem cells

There is strong evidence that cancer stem cells (CSCs), also known as tumor-initiating cells, which consist of a small subset of cells within a tumor, are responsible for the development of tumors and resistance to therapeutic agents and radiotherapy (70-74). As shown experimentally, these cells are characterized by cell surface markers or aldehyde dehydrogenase activity and have the capacity to self-renew and generate a tumor (70-80). The ability to isolate CSCs has provided the elucidation of molecular and genetic characterization of these cells. Recently, it has been shown that the Notch signaling pathway is dysregulated in this population of cells, among many different tumor types (81-86). van Es et al. completed some of the initial work in identifying Notch as an important regulator of stem cells (46). Utilizing a Cre-Lox system, they deleted the RBP-J allele and demonstrated that disruption of the Notch pathway resulted in an increase in goblet cells in the transit-amplifying compartment, which is normally rapidly proliferating. These results show that the Notch pathway is fundamental for the maintenance of undifferentiated cells within the crypt compartment. Additionally, they investigated the role of the Notch signaling pathway in the formation of adenomas in the intestine and colon in an $APC^{-/-}$ model. Pharmacological inhibition of the Notch pathway resulted in an increase in terminally differentiated goblet cells, reducing tumor burden in these animals. Taken together, these results demonstrate that the Notch pathway is required for self-renewal of stem/progenitor cells and that treatment with a γ-secretase inhibitor in an intestinal adenoma model was beneficial.

Several other studies in preclinical models of colorectal cancer have evaluated the Notch pathway in tumor-initiating cells. A study by Hoey et al. (86) developed a Delta4 antibody and assessed the effects of inhibiting Delta4 on tumor volume and recurrence in a colorectal cancer explant model. The treatment resulted in a decrease in tumor volume. Interestingly, the addition of irinotecan to the Delta4 antibody had additive effects on tumor volume and significantly altered the regrowth of tumors. Isolation and evaluation of the CSCs within this tumor revealed a decrease in the Notch signaling pathway in the Delta4 and the Delta4 + irinotecan groups. Furthermore, another study showed that Notch signaling was upregulated in colon cancer-initiating cells (CCICs) when compared to normal colon tissue. The Notch pathway prevented apoptosis in this population of cells through inhibition of the cyclin-dependent kinase p27. Use of the γ-secretase inhibitor DAPT increased apoptosis of the CCIC population and goblet cell lineage markers (85). Both studies demonstrated the importance of the Notch pathway in tumor-initiating cells and in potentiating cancer of the colon.

In addition to the Notch pathway playing an important role in CSCs in colorectal cancer, this pathway has also been shown to play a role in CSCs of other tumor types. In particular, glioblastoma and medulloblastoma CSCs have been identified to have higher levels of Notch activity. Fan et al. demonstrated that treatment with a γ-secretase inhibitor resulted in a reduction of the CSC markers CD133, nestin, polycomb complex protein BMI-1 and oligodendrocyte transcription factor 2 (Olig02), and neurosphere formation (84). Additionally, treatment of neurospheres prior to injection in mice prevented tumor formation, indicating that blockade of the Notch pathway attenuated the tumorigenic capacity of these cells. Another study evaluating glioma stem cells demonstrated that γ-secretase inhibitors sensitize the CSC population to radiation therapy (83). In pancreatic cancer, γ-secretase inhibitors reduced the population of ALDEFLUOR®-positive cells and the growth of tumors when these cells were injected in vivo (82). Furthermore, in breast cancer, Notch 4 signaling was eightfold higher in the $ESA^{+}/CD44^{+}/CD24^{low}$ stem cell population and blockade of the pathway decreased tumor formation in vivo (81). Strong evidence exists from many different tumor types that dysregulation of the Notch pathway occurs in the CSC/tumor-initiating population and that treatment with a γ-secretase inhibitor or antibody specific to the Notch ligand Delta4 either alone or in combination with other drugs or radiation may be beneficial for reducing tumor burden and disease recurrence.

Linking Notch to angiogenesis

Genetic studies have revealed that the Notch signaling pathway is an important modulator of vascular development and tumor angiogenesis. Deletion of Notch 1 or Delta4 results in embryonic lethality associated with vascular dysfunction (87). Recently, a study by Hellström et al. demonstrated that the activation of the Notch 1 receptor by Delta4 is an essential regulator of the number of tip cells and controls the branching and sprouting of vessels in the mouse retina. Genetic deletion of Notch 1 or Delta4 resulted in an increased number of tip cells, suggesting that Delta4/Notch 1 in endothelial cells in response to vascular endothelial growth factor (VEGF) controls tip sprouting and branching (88). Tumors that require new blood vessels to support the growth of the tumor secrete proangiogenic factors such as VEGF to recruit endothelial cells. There is accumulating evidence that the Notch signaling pathway interacts with VEGF to regulate this process (89). In particular, VEGF has been shown to upregulate the expression of Delta4 in endothelial cells through the activation of the PI3K pathway. Additionally, a study by Noguera-Troise et al. (90) showed that VEGF increased the expression of Delta4 in tumor vessels. Inhibition of Delta4 enhanced tumor vascularity, as evidenced by an increase in angiogenic sprouting and branching. Although an increase in vascular density was evident in these tumors through Delta4 blockade, this was associated with nonproductive angiogenesis that ultimately affected tissue perfusion and increased hypoxia and growth of tumors.

NOTCH PATHWAY INHIBITION IN HUMAN DISEASE

As discussed above, activation of the Notch pathway contributes to tumorigenesis, maintenance of the CSC population and angiogenesis in a number of cancers. Robust emerging evidence suggests that evolutionarily conserved developmental pathways such as Notch, hedgehog and Wnt are multifunctional and control key "nodes" in cancer cell signaling; therefore, inhibition of these pathways may

have far-reaching therapeutic effects. Also, these developmental pathways are critical for maintenance of cancer stem cells. These cells are characterized by properties such as indefinite, slow proliferation, resistance to toxic agents and an ability to remain in a near-quiescent state to produce recurrences and metastases. Thus, eradication of these cells is essential for a complete cure (91-93). Finally, the interaction between cancer cells and surrounding stroma has gained recent attention as a key factor and therapeutic target in tumor progression. Emerging evidence supports the existence and importance of such bidirectional communication between tumor and stromal cells through these developmental pathways (93). Therefore, these pathways are attractive targets in cancer therapy.

Several features of the Notch pathway have unique relevance for targeting purposes: 1) the activation of Notch receptors by ligands does not lead to an amplification step by enzymes such as kinases, which suggests that the downstream effects of this pathway may be very dose-sensitive to inhibitors (94); 2) Notch signaling typically leads to gene regulation in short pulses, and thus, sustained inhibition of this pathway may not be necessary (95). These two features suggest that Notch pathway inhibition need not be complete or continuous, and these considerations are important in attaining efficacy without causing excessive toxicity. However, it should also be remembered that Notch signaling is exquisitely context- and tissue-dependent; therefore, systemic therapy with γ-secretase inhibitors will likely have a multitude of effects with potential for unexpected side effects. It would also be prudent and more effective to identify context-specific targets, develop selective delivery of Notch inhibitors and develop drug combinations against pathways in cross-talk with Notch.

The Notch pathway offers targets for inhibition at multiple levels and compounds against these targets are in various stages of development. These include decoy ligands such as soluble Delta4, antibodies against ligands or Notch receptors to inhibit their activation, inhibitors of TACE and γ-secretase that are involved in cleavage of the activated Notch receptor to release the active NICD, mastermind-like protein 1 (Mam-1, *MAML1*)-stapled peptides interfering with functioning of the Notch nuclear co-activator Mam-1 and siRNA, miRNA approaches to inhibit post-transcriptional processing of gene products. These various approaches have been reviewed elsewhere (91, 92, 96-98). Antibodies against Delta4 ligand and Notch 1 receptor and γ-secretase inhibitors are the classes of Notch pathway inhibitors currently in clinical trials. Of these, γ-secretase inhibitors are by far in the most advanced stage of clinical development, which is not surprising given initial, extensive efforts at developing these agents for the treatment of Alzheimer's disease (AD) (91, 95).

γ-Secretase is the final protease involved in the sequential cleavage of amyloid precursor proteins to release β-amyloid (Aβ) peptides that aggregate into the neurotoxic senile plaques characteristic of AD. Therefore, several γ-secretase inhibitors were developed for AD therapy that showed promising preclinical results in a mouse model of AD, with reduction in brain Aβ levels (99). LY-450139 (semagacestat), an oral γ-secretase inhibitor, when evaluated in a phase II trial was well tolerated and also showed an initial reduction in plasma $A\beta_{40}$ concentrations. However, this reduction in plasma $A\beta_{40}$ was biphasic, with a subsequent increase in levels, and was also not associated with significant reductions in spinal fluid Aβ or improvements in cognitive or functional measures after therapy for 14 weeks. Based on these results, 2 randomized, placebo-controlled phase III trials of semagacestat in over 2,600 patients with mild to moderate AD were initiated. However, these trials were prematurely halted after a pre-planned interim analysis showed that patients in the treatment arm had a significantly worse decline in cognitive function and were also at increased risk of skin cancer as compared to those in the control arm (100, 101). As discussed above, the increased risk of skin cancer in this trial may potentially be related to the tumor suppressor function of Notch 1 in keratinocytes. Although these results suggest that γ-secretase may not be a valid therapeutic target in AD, these efforts have paved the way for the rapid development of γ-secretase inhibitors in oncology.

NOTCH TARGETING BY γ-SECRETASE INHIBITORS IN CANCER

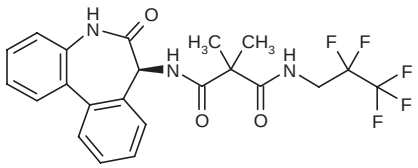
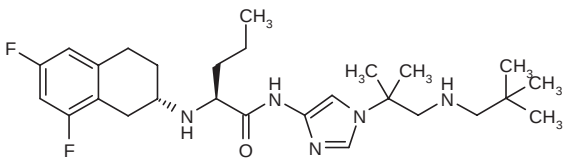
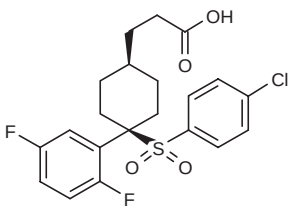
There are several γ-secretase inhibitors in clinical development either as single agents or in combination with other targeted or cytotoxic agents. Here, we review the salient preclinical and clinical data on γ-secretase inhibitors in development. Properties of γ-secretase inhibitors in clinical use are summarized in Table I. Completed and ongoing clinical trials of γ-secretase inhibitors are summarized in Table II. Clinical trial information was obtained from www.clinicaltrials.gov.

RO4929097

RO4929097 (Hoffmann-La Roche, Inc.) is an orally active, potent and selective inhibitor of γ-secretase derived from LY-411575. It has an IC_{50} in the low nanomolar range in cell-free and cellular assays and > 100-fold selectivity for γ-secretase with respect to 75 other proteins. A variety of tumor cell lines, including colon, breast, lung, melanoma and pancreatic cancer cells, treated with this agent showed a flattened, slow-growing and less transformed phenotype, without inhibition of proliferation or induction of apoptosis. In vivo studies showed activity in seven of eight murine xenograft models, including colon, pancreatic and non-small cell lung cancers, without any overt toxicity. Real-time polymerase chain reaction and microarray analysis of these tumors showed reduced expression of angiogenic genes and increased tumor differentiation, consistent with the known antitumor effects of Notch pathway inhibition. Effects of RO4929097 on cancer stem cells were not evaluated in this study due to limitations of the models used (102). Interestingly, efficacy was seen with both continuous and intermittent dosing, and furthermore, the effects persisted after cessation of treatment, possibly due to effects on cancer stem cells. Other preclinical studies in cell lines and murine xenograft models of melanoma (103) and pediatric gliomas (104) showed similar results on cell phenotype and tumor growth.

Based on these results, a multicenter phase I study of RO4929097 was initiated that, according to preliminary results presented at the 2010 ASCO Annual Meeting, had accrued 89 patients to receive 2 intermittent dosing schedules: either days 1-3 and 8-10 every 3 weeks (3-270 mg/day) or days 1-7 every 3 weeks (3-135 mg/day). Overall, this agent was well tolerated, with common adverse events including fatigue, nausea, diarrhea, hypophosphatemia, pruritus and rash, 92% being grade 1/2 and no grade 4 toxicities. However,

Table I. *Properties of γ-secretase inhibitors in clinical use.*

Compound	Structure	MTD	Clinical trials	Toxicities/AEs
RO4929097		MTD not reached but 20 mg, 3 days on, 4 days off is the RP2D based on PK studies	Phase I, II in adult solid tumors, myeloma, pediatric CNS tumors, leukemia and lymphoma	Fatigue, nausea, emesis, diarrhea, hypophosphatemia, pruritus and rash
PF-03084014		Phase I trial ongoing	Phase I in adult solid tumors and T-cell ALL	Nausea, diarrhea, anorexia and hypophosphatemia
MK-0752		260 mg/m^2 3 days on, 4 days off in pediatric population	Phase I, II in adult solid tumors, non-Hodgkin's lymphoma and pediatric CNS tumors	Intolerable constipation, diarrhea, nausea, abdominal cramping, elevated liver enzymes with continuous dosing, hypokalemia, lymphopenia with intermittent dosing

MTD, maximum tolerated dose; AEs, adverse events; RP2D, recommended phase II dose; PK, pharmacokinetics.

Table II. *Oncology clinical trials with γ-secretase inhibitors.*

Trial design	Phase, status	Comments
Completed trials of RO4929097 (R)		
R in advanced solid tumors (N = 89)	I	MTD not reached, RP2D is 20 mg, 3 days on, 4 days off due to autoinduction; R well tolerated with low-grade toxicities, mostly fatigue, nausea, emesis, diarrhea, hypophosphatemia, pruritus and rash (105)
R in metastatic colorectal cancer (N = 37)	II	No objective responses, median progression-free survival 8 weeks, median overall survival 6 months (107)
Ongoing single-agent trials of RO4929097 (R)		
R in solid tumors to evaluate PK (N = 40)	Ongoing(ClinicalTrials.gov Identifier NCT01218620)	To assess effects of: 1) inducers/inhibitors of CYP3A4 on PK of R, including R; 2) R on PK of CYP450 substrates; 3) specific polymorphisms of CYP450 on R PK
R in metastatic renal cell carcinoma refractory to VEGF/VEGFR therapy (N = 39)	II, ongoing (ClinicalTrials.gov Identifier NCT01141569)	PD studies include evaluation of Notch biomarkers
R in recurrent malignant gliomas (N = 22)	I, ongoing (ClinicalTrials.gov Identifier NCT01269411)	Proposed studies include evaluation of effects of R on Notch pathway, on brain tumor-initiating cells in vitro and on p75 neurotropin receptor cleavage
R in neoadjuvant stages IIIB, IIIC and IV resectable melanoma (N = 15)	II, ongoing (ClinicalTrials.gov Identifier NCT01216787)	PD studies include Akt-mediated downstream markers, micro-RNA signatures from pre- and post-treatment tissue biopsies, serum angiogenesis markers

Continued

Table II. *Cont. Oncology clinical trials with γ-secretase inhibitors.*

Trial design	Phase, status	Comments
R in residual multiple myeloma post-autologous transplant after melphalan conditioning (N = 37)	II, ongoing (ClinicalTrials.gov Identifier NCT01251172)	PD studies include Notch receptor expression, ligand expression, microvessel density and VEGFR-1 expression in pre- and post-treatment bone marrow biopsies, serum markers of angiogenesis
R in recurrent or progressive glioma as palliative or neoadjuvant therapy (R = 60)	II, ongoing (ClinicalTrials.gov Identifier NCT01122901)	Patients with resectable disease treated for with a short course prior to surgery. Pre-, post-treatment biopsies used for evaluating Notch pathway components and targets
R in advanced solid tumors (R = 30)	I, ongoing (ClinicalTrials.gov Identifier NCT01198184)	Expanded cohorts in recurrent / metastatic endometrial and renal cell carcinomas
R in metastatic NSCLC after completion of prior front-line cytotoxic-based chemotherapy (N = 30)	II, ongoing (ClinicalTrials.gov Identifier NCT01193868)	PD studies include changes in Notch pathway markers, correlation of EGFR-activating mutations with Notch and stem cell marker expression
R in metastatic melanoma (N = 72)	II, ongoing (ClinicalTrials.gov Identifier NCT01120275)	Secondary outcomes include effects on T-cell function
R as neoadjuvant therapy in resectable pancreatic cancer (N = 30)	I, ongoing (ClinicalTrials.gov Identifier NCT01192763)	PD studies include HES-1 expression, proportion of cancer stem cells post-treatment
R in previously treated metastatic pancreatic cancer (N = 36)	II, ongoing (ClinicalTrials.gov Identifier NCT01232829)	Proposed studies include pharmacogenetics-based evaluation of R exposure, PD studies on pre- and post-treatment biopsies for Notch pathway and targets
R in advanced, metastatic or recurrent triple-negative breast cancer (N = 50)	II, ongoing (ClinicalTrials.gov Identifier NCT01151449)	PD studies include evaluation of Notch pathway in pre- and post-treatment biopsies
R in recurrent or metastatic epithelial ovarian, fallopian tube or primary peritoneal cancers (N = 37)	II, ongoing (ClinicalTrials.gov Identifier NCT01175343)	PD studies include evaluation of Notch biomarkers and ascitic fluid circulating tumor cells
R in advanced solid malignancies (N= 92)	I, ongoing (ClinicalTrials.gov Identifier NCT01096355)	Dose escalation phase with six different intermittent dosing schedules. PK and tumor biopsy-based PD studies to be correlated to define optimal biological dosing schedule
R in advanced solid tumors (N = 125)	I, ongoing (ClinicalTrials.gov Identifier NCT00532090)	Dose escalation phase with two intermittent and one continuous dosing schedules
Ongoing combination trials of RO4929097 (R)		
R + cetuximab vs. cetuximab + placebo in metastatic colorectal cancer (N = 132)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01198535)	Phase I dose escalation with cetuximab + R and open to unselected patients. Dose expansion and phase II in wild-type *KRAS* patients only with 2:1 randomization in phase II
R + radiation in patients with brain metastases (N = 142)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01217411)	Phase I: R + whole brain radiation (> 4 lesions) or with stereotactic radiation (< 4 lesions) in patients with brain metastases; phase II: limited to ER-negative breast cancer
Exemestane ± R in ER-positive, HER2/neu-negative metastatic breast cancer (N = 104)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01149356)	Phase II: patients randomized to exemestane with or without R; stratified per menopausal status, visceral disease
FOLFOX + bevacizumab ± R in first-line metastatic colorectal cancer therapy (N = 98)	II, ongoing (ClinicalTrials.gov Identifier NCT01270438)	PD studies include effects on Notch, Ras pathway and colorectal cancer stem cells
R + paclitaxel + carboplatin as neoadjuvant therapy in triple-negative breast cancer (N = 18)	I, ongoing (ClinicalTrials.gov Identifier NCT01238133)	Will also evaluate PK of R and paclitaxel when given as combination
R ± dexamethasone in pediatric solid tumors, lymphoma and T-cell leukemia (N = 129)	I/II active, not recruiting (ClinicalTrials.gov Identifier NCT01088763)	PD studies include effects of R on HES-1, baseline jagged1, jagged2, cleaved Notch 1, HES-1 and HES-5, amplification of Notch 1, Notch 2, changes in PET scans

Continued

Table II. *Cont. Oncology clinical trials with γ-secretase inhibitors.*

Trial design	Phase, status	Comments
R + letrozole as neoadjuvant therapy in hormone receptor-positive breast cancer (N = 28)	I, ongoing (ClinicalTrials.gov Identifier NCT01208441)	PD studies include Notch pathway, proliferation, angiogenesis, stromal cell infiltration/pathways and comprehensive genomic analysis in tumor tissues
R + cisplatin + vinblastine + temozolomide in melanoma	I/II, ongoing (ClinicalTrials.gov Identifier NCT01196416)	PK, PD studies planned (further details unavailable)
R ± GDC-0449 (hedgehog inhibitor) in sarcoma (N = 120)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01154452)	In phase II, patients randomized to R alone or R + GDC-0449 stratified according to sarcoma type. Pre- and post-treatment tumor biopsies planned for PD studies
R + erlotinib in stages IIB, IV or recurrent non-small cell lung cancer (N = 54)	I, ongoing (ClinicalTrials.gov Identifier NCT01193881)	PD studies include baseline IHC and reverse-phase protein array expression of Notch 1, 2, 3 and 4. Patients stratified according to EGFR-activating mutation status
R + capecitabine in solid tumors (N = 40)	I, ongoing (ClinicalTrials.gov Identifier NCT01158274)	PD studies include PCR evaluation of HES-1, -3, -5 and HEY1 and HEY2
R + cediranib in patients with advanced solid tumors (N = 50)	I, ongoing (ClinicalTrials.gov Identifier NCT01131234)	Cediranib (AZD-2171; AstraZeneca) is an inhibitor of VEGF receptor tyrosine kinases
R + bicalutamide in previously treated prostate cancer (N = 78)	II, ongoing (ClinicalTrials.gov Identifier NCT01200810)	Placebo-controlled trial with patients randomized to R therapy or placebo
R + temozolomide + radiation in newly diagnosed malignant gliomas (N = 24)	I, ongoing (ClinicalTrials.gov Identifier NCT01119599)	PD studies include angiogenesis markers such as vascular E-cadherin, CD146, CD31, VEGF ligands and receptors, and pericyte markers, DCE-MRI perfusion and Notch inhibition in hair follicles
R + gemcitabine in advanced solid tumors (N = 28)	I, ongoing (ClinicalTrials.gov Identifier NCT01145456)	≥ 10 patients with pancreatic cancer to be accrued
Bevacizumab vs. bevacizumab + R in progressive or recurrent malignant glioma (N = 112)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01189240)	PD studies in archival tissue for Notch pathway analysis
Completed trials of MK-0752 (M)		
M in adult and pediatric T-cell ALL and other leukemias (N = 8)	I	DLT of grade 3/4 diarrhea at 300 mg/m^2; 1 patient with T-cell ALL with short-lived partial response in mediastinal mass (119)
M in advanced breast cancer and other solid tumors (N = 7)	I	Intolerable grade 3 diarrhea, constipation, nausea, fatigue and abdominal cramping with continuous dosing of 450-600 mg daily (112)
M in pediatric recurrent or refractory nervous system tumors (N = 23)	I	At a dose 260 mg/m^2/day (RP2D), DLTs were grade 3 elevation in liver enzymes. Other grade 3 toxicities were hypokalemia and lymphopenia (113)
Ongoing trials of MK-0752 (M)		
M as neoadjuvant therapy with anti-endocrine agents in ER-positive resectable breast cancer (N = 20)	I, ongoing (ClinicalTrials.gov Identifier NCT00756717)	Post-menopausal women to be treated with letrozole, pre-menopausal women to receive tamoxifen along with M
M + ridaforolimus in advanced solid tumors, non-Hodgkin's lymphoma (phase I only) (N = 124)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01295632)	Phase I to allow non-Hodgkin's lymphoma; phase II limited to prostate, breast, glioblastoma multiforme and ovarian cancers
M + gemcitabine in metastatic pancreatic cancer (N = 60)	I/II, ongoing (ClinicalTrials.gov Identifier NCT01098344)	PD studies include changes in Notch pathway via IHC and/or RT-PCR in tumor biopsies and hair follicles, changes in PET scan
Dalotuzumab + MK-2206 or dalotuzumab + M in advanced tumors with dose expansion cohorts in NSCLC, colon cancer (N = 78)	I, ongoing (ClinicalTrials.gov Identifier NCT01243762)	Dalotuzumab is a humanized monoclonal IGF-I receptor antibody; MK-2206 is an allosteric Akt inhibitor; patients with NSCLC and colon cancer with high IGF-I receptor or mucinous colon cancer to be enrolled in a dose expansion phase

Continued

Table II. *Cont. Oncology clinical trials with γ-secretase inhibitors.*

Trial design	Phase, status	Comments
M + docetaxel in advanced, anthracycline-resistant breast cancer (N = 30)	I/II, active but not recruiting (ClinicalTrials.gov Identifier NCT00645333)	Docetaxel therapy supported with pegfilgrastim
M in pediatric central nervous system malignancies (N = 34)	I, active but not recruiting (ClinicalTrials.gov Identifier NCT00572182)	Two different intermittent dosing regimens (once weekly, 3 days on/4 days off of 28-day cycles) to be evaluated
M in advanced breast cancer and other solid tumors (N = 50)	I, active but not recruiting (ClinicalTrials.gov Identifier NCT 00106145)	Multiple continuous and intermittent dosing regimens to be explored
PF-03084014 (P)		
P with or without dexamethasone in advanced solid tumors and T-cell ALL (N = 60)	I, ongoing (ClinicalTrials.gov Identifier NCT00878189)	PD studies include NICD and Notch 1 levels

MTD, maximum tolerated dose; PK, pharmacokinetics; PD, pharmacodynamics; NSCLC, non-small cell lung cancer; ER, estrogen receptor; ALL, acute lymphocytic leukemia; DLT, dose-limiting toxicity; RP2D, recommended phase II dose; NICD, Notch intracellular domain; IHC, immunohistochemistry.

dose escalation was limited by reversible, dose-related autoinduction of metabolism of RO4929097. Although there were no dose-limiting toxicities and the maximum tolerated dose was not reached, based on pharmacokinetic studies, the recommended phase II dose was 20 mg p.o. 3 days on, 4 days off continuously. Eleven patients had an FDG-PET response including a PET complete response (CR) in a melanoma patient. Other patients who had prolonged clinical benefit included four sarcoma and three ovarian carcinoma patients (105). Interestingly, the patients who had clinical benefit in this trial had low levels of IL-6 and IL-8 at baseline. This is consistent with preclinical data at both the tissue culture and xenograft levels, which suggested that tumors overexpressing IL-6 and IL-8 are resistant to RO4929097. This is possibly because IL-6 and IL-8 have proangiogenic effects during tumor progression and tumors overexpressing these interleukins are resistant to the antiangiogenic effects of RO4929097 (106).

Over 30 phase I and phase II trials are currently under way with RO4929097 either alone or in combination with other agents in multiple solid tumors, including breast, melanoma, colorectal and CNS tumors, in addition to T-cell ALL. Based on results of microarray analysis showing upregulation of the Notch 1 receptor in colorectal cancer, a phase II trial of RO4929097 in 37 unselected patients with chemorefractory metastatic colorectal cancer was performed, which failed to show any objective radiographic responses (primary objective). Progression-free survival was 8 weeks and median overall survival was 6 months (107). Although the results of this phase II trial are disappointing, it should be remembered that patients were unselected for potential biomarkers and were also treated with single-agent RO4929097. Experience with other targeted agents such as EGFR antibodies in colorectal cancer has shown the importance of biomarker-based preselection of patients and also that outcomes are typically better with combination therapy.

PF-03084014

PF-03084014 (Pfizer) is an orally active, small-molecule, reversible, noncompetitive γ-secretase inhibitor with a low IC_{50} that was initially evaluated for its ability to decrease $A\beta_{40}$ production (108). However, a subsequent study in preclinical models of T-cell ALL established its antitumor effects. In this study, PF-03084014 was noted to cause inhibition of several T-cell ALL cell lines associated with a reduction in NICD levels and downregulation of Notch target genes. However, in mice treated with higher doses of PF-03084014, diarrhea associated with weight loss was observed. This toxicity was largely avoided by an intermittent dosing schedule of 7 days on/7 days off, without loss of efficacy (109). Prior studies have suggested that the gastrointestinal toxicities of γ-secretase inhibitors are due to Notch pathway inhibition in the precursor cells of intestinal crypts, leading to aberrant differentiation and goblet cell hyperplasia in the intestinal epithelium (46). Another study showed that concomitant treatment with steroids leads to protection from the gastrointestinal toxicities of γ-secretase inhibitors by preventing goblet cell metaplasia. The protective effects of steroids were further confirmed in other preclinical studies of PF-03084014 (109). Furthermore, inhibition of γ-secretase leads to reversal of steroid resistance in T-cell ALL cell lines, suggestive of synergistic effects when γ-secretase inhibitors are combined with steroids (110).

Based on these results, a phase I study of PF-03084014 (ClinicalTrials.gov Identifier NCT00878189) with or without steroids is ongoing in adult patients with advanced solid tumors or T-cell ALL. Preliminary results after accrual of 16 patients showed that this agent was well tolerated, mainly associated with low-grade nausea, diarrhea and anorexia. Grade 3 toxicities observed included diarrhea and hypophosphatemia. One patient developed an anaphylactic reaction after dosing with this drug followed by i.v. morphine; one patient with papillary thyroid cancer had a CR, another subject with a sporadic desmoid tumor had a partial response and six other patients had stable disease (111).

MK-0752

MK-0752 (Merck Research Laboratories) is another orally active, small-molecule γ-secretase inhibitor with an IC_{50} of 55 nM. In an initial phase I trial in metastatic breast cancer, the agent showed poor tolerability with continuous dosing at 450-600 mg/day p.o., with

significant toxicities, predominantly fatigue. Given the significant toxicities, intermittent dosing will be evaluated in future cohorts in this trial (112). Another smaller phase I trial in eight patients (including two pediatric patients) also explored continuous dosing and showed dose-limiting diarrhea at 300 mg/m^2. A more recent phase I trial of this agent in 23 pediatric patients with refractory CNS malignancies with an intermittent dosing schedule showed better tolerability. It did not show any grade 4 toxicities and grade 3 toxicities included lymphopenia and hypokalemia. The recommended phase II dose for this agent according to this trial was 260 mg/m^2/day for 3 days every 7 days of a 28-day cycle (113).

Other molecules

Another γ-secretase inhibitor currently in preclinical development is BMS-708163 (Bristol-Myers Squibb). Also, several compounds, including nonsteroidal antiinflammatory drugs (NSAIDs) such as ibuprofen, sulindac sulfide and indomethacin, and the related compound flurbiprofen (*R*-tarenflurbilin), have been shown to negatively modulate the activity of the γ-secretase enzyme complex (114). This is in line with epidemiological data suggesting decreased rates of AD with long-term use of NSAIDs (115-117). However, disappointingly, a large, randomized phase III trial of tarenflurbilin in patients with mild AD failed to show any clinical benefit (118). Apart from a single placebo-controlled phase II trial of tarenflurbilin (ClinicalTrials.gov Identifier NCT00045123) after definitive therapy in localized prostate cancer (initiated in 2002 and the current status of which is unknown), there are no current clinical trials of γ-secretase modulators. The role of these agents in oncology therefore needs to be defined.

CONCLUSIONS

The Notch pathway, along with other developmental pathways like the hedgehog and Wnt pathways, is evolutionarily conserved and has been shown to be reactivated in multiple tumor types. This pathway is a novel and promising target for cancer therapy given its role in cancer stem cell maintenance, cell differentiation and neoangiogenesis. Accumulating evidence supports the critical role of this pathway in a multitude of cancers through distinct and even opposite effects that appear to be context- and tumor type-dependent. Complete understanding of this pathway and its functions is thus critical to improve therapeutic options with Notch pathway inhibitors. Multiple Notch pathway inhibitors are currently in development, of which γ-secretase inhibitors are in early-phase clinical trials in advanced solid tumors and hematological malignancies, such as ALL and myeloma. However, current γ-secretase inhibitors do not appear to be specific to the Notch pathway and may inhibit γ-secretase and other enzymes at various sites, leading to unwanted toxicities. Therefore, the development of newer classes of compounds with higher specificity for the Notch pathway is warranted. Also, the results of a recent phase II trial of a γ-secretase inhibitor in unselected metastatic colorectal cancer patients failed to show any objective responses. Although disappointing, this is not unexpected and is in line with experience with clinical trials evaluating other targeted agents as monotherapy in unselected populations. Therefore, it is vital to identify and validate biomarkers for γ-secretase inhibitor therapy early in the drug development process. Furthermore, appropriate combination of γ-secretase inhibitors with inhibitors of pathways interacting with the Notch pathway may prove to be synergistically beneficial.

DISCLOSURES

Dr. Messersmith has received research support from Pfizer and Roche. The other authors state no conflicts of interest.

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