γ-Secretase Inhibitor Prevents Notch3 Activation and Reduces Proliferation in Human Lung Cancers

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Abstract

Notch receptors are key regulators of development by controlling cell-fate determination in many multicellular organisms. Genes that are important for normal differentiation play a role in cancer when their normal functions became dysregulated. Notch signaling has been shown to promote and maintain survival of many types of cancers, and we previously have shown that Notch3 plays an important role in lung cancer. In this study, we showed that a high percentage of lung cancer lines expressed Jagged1, Notch receptors, and their transcriptional target genes (*HES1, Hey1*), suggesting that the Notch pathway plays an important role in lung cancer biology. Thus, inhibition of Notch receptor activation represents a compelling treatment strategy. Notch activation requires proteolytic cleavage of the receptor by γ-secretase protein complex. In this study, we determined the ability of MRK-003, a γ-secretase inhibitor, to inhibit Notch3 signaling, growth, and apoptosis of lung cancer cell lines *in vitro* and *in vivo* using mouse xenograft models. We also found that MRK-003 inhibited Notch3 signaling, reduced tumor cell proliferation, inhibited serum independence, and induced apoptosis. This drug had no effect when Notch3 expression was knocked down using small interfering RNA (siRNA), suggesting that the observed effects were mediated by specific action on this receptor. In conclusion, these results support the hypothesis that inhibition of Notch activation using a γ-secretase inhibitor represents a potential new approach for the targeted therapy of lung cancer. [Cancer Res 2007;67(17):8051–7]

Introduction

Notch receptors are single-pass, transmembrane receptors whose function is important for normal cell-fate determination in all known multicellular organisms. In mammals, there are four Notch receptors, Notch 1 to 4. Notch receptors signal through binding with their ligands, Jagged 1 and 2 and Delta-like-1, Delta-like-3, and Delta-like-4. Upon ligand binding, the Notch receptor undergoes a series of proteolytic cleavages, resulting in the release of the intracellular domain, which forms a nuclear complex with the DNA-binding protein CSL (CBF1, Sel, Lag-1). This interaction then leads to transcription of the bHLH transcription factors, *Hairy-enhancer of Split* (*HES*) and related genes (*Hey*; ref. 1). Although the canonical Notch pathway involves signaling through *HES* and related genes, Notch receptors are known to crosstalk with other pathways involved in oncogenesis, suggesting the possibility that multiple other pathways are regulated by Notch receptors, and that these interactions are important in maintaining the oncogenic state (2–4).

All four members of the Notch family have been implicated in human cancers (5–10). However, depending on the cellular context, Notch family members can function as oncogenes or tumor suppressors (11). We first linked the dysregulation of the Notch3 pathway to human lung cancer through the observation of an upstream chromosome translocational breakpoint in a tumor from a nonsmoker (6). Subsequently, we observed overexpression of Notch3 in about 40% of resected, non–small cell lung cancers (NSCLC). Through Serial Analysis of Gene Expression (SAGE) profiling data on NSCLC, other investigators show high expression of Hey1 and HeyL, suggesting that the activation of the Notch pathway represents an important role of Notch signaling in lung cancer tumorigenesis (12). Furthermore, we have shown that inhibiting this pathway in human lung tumors results in the loss of the malignant phenotype *in vitro*, in the increased dependency on exogenous growth factors, and in greater cytotoxicity when combined with an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (13). Taken together, these observations suggest that the inhibition of Notch activation either alone or in combination represents a potential therapeutic strategy in the clinical setting.

Activation of the canonical Notch pathway involves a series of proteolytic processing steps following ligand binding. The final cleavage of Notch receptor by the γ-secretase protein complex releases the Notch intracellular domain whose translocation to the nucleus is thought to be required for target gene transcription (14). Any pharmacologic intervention that interferes with the activity of these proteases can thus theoretically prevent tumor growth in a Notch-dependent cancer. Presenilin-1 and presenilin-2 are polytopic transmembrane proteins, and they function as part of the γ-secretase protein complex, which include cofactors nicastrin, APH-1, and PEN-2 (15). This protein complex is also known to be essential in the normal processing of amyloid β-peptides (Aβ). These peptides are derived from the proteolytic processing of the β-amyloid precursor protein (APP) through an intermediate fragment (C99) by γ-secretases. Abnormal accumulation of amyloid β-peptides (Aβ) with formation of amyloid plaques is believed to be the pathogenesis of Alzheimer's disease. Indeed, a familial form of Alzheimer's disease (FAD) is a result of mutations in presenilin-1 or presenilin-2 (16). Given the connection between Alzheimer's disease and γ-secretase, there is a great interest in pursuing compounds that can inhibit this protein complex and thus alleviate progression in Alzheimer's disease.

Not surprisingly, because γ-secretase protein complex is also necessary for Notch processing, γ-secretase inhibitors developed as potential treatment for Alzheimer's disease also block Notch

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activation and induce apoptosis in multiple cancer cell lines (17–20). *In vivo*, these compounds inhibit angiogenesis and tumor growth (21). Furthermore, some γ-secretase inhibitors are currently in phase I trials for patients with metastatic or locally advanced breast cancer and for patients with T-cell acute leukemias (22, 23). Given the data suggesting a role for Notch in lung cancer, we examined whether targeting of the Notch pathway using a γ-secretase inhibitor, MRK-003, can block Notch signaling in NSCLC, induce apoptosis, and reduce tumor growth both *in vitro* and in tumor xenografts.

Materials and Methods

Cell lines and inhibitors. HCC2429 was established as previously described (6). NSCLC cell lines HCC461, HCC193, HCC95, HCC15, HCC827, HCC44, and HCC78 were provided by Drs. John D. Minna and Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). The remaining lung cancer cell lines were obtained from American Type Culture Collection (ATCC). All lung cancer cell lines were maintained in RPMI with 10% FCS and analyzed at 80–90% confluency. Tyrosine kinase inhibitor to EGFR AG1478 was obtained from Calbiochem. The γ-secretase inhibitor MRK-003, its formulation, and *in vivo* dosing were provided by Merck & Co., Inc. (20).

Antibodies and Western blot experiments. Notch3 was detected using a goat polyclonal antibody at 1:1,000 dilution from Orbigen, Inc. Notch2 (C651.6DbHN, developed by Artavanis-Tsakonas) and Notch4 (2423) antibodies from the Developmental Studies Hybridoma Bank at the University of Iowa and Cell Signaling Technology, Inc. were used at dilutions of 1:200 and 1:500, respectively. Notch1 (C-20) and Jagged1 (H-114) were obtained from Santa Cruz Biotechnology and used at 1:100 and 1:200 dilutions. Antibodies to phospho–extracellular signal-regulated kinase (phospho-ERK), total ERK, phospho–Bcl-2, Bcl-2, Bcl-xL, and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling and used in accordance with the manufacturer's instructions. For Western blot analysis of ERK activation, the cells were maintained in serum-free medium for 24–48 h before serum stimulation. The γ-secretase inhibitor was also added 24 h before serum stimulation. Cells were harvested after the designated time points.

Stable transfectants and small interfering RNAs. Notch3-overexpressing cell line HCC2429 were transfected with DNA vector-based small interfering RNA (siRNA) using Genscript siRNA Expression Vector and LipofectAMINE 2000 (Invitrogen). The target insert is 5′-CACCUAUAACUGCCAGUGC-3′. Stable clones were selected using hygromycin. Clones 5, 6, and 8 retained Notch3 expression, whereas clones 12, 15, 17, and 20 lost Notch3 expression. Transient knockdown of Notch3 was done using siRNA with above sequences, synthesized by Qiagen.

Apoptosis assays. Transfected cells were plated at a density of 5,000 cells per plate using 35-mm Petri dishes and suspended in 0.4% agar containing 10% FCS RPMI and 50 μg/mL of G418 selective antibiotic over a 0.8% base agar. The plates were incubated at 37°C and 5% CO_2 humidified chamber for 14 days. The colonies were counted on the Omnicon Tumor Colony Analyzer. Changes in proliferation were determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. Each experiment was repeated at least thrice with similar results. Apoptosis was measured following 48 h of serum starvation, unless otherwise indicated, using the APO-BRDU kit (Pheonix Flow Systems).

Quantitative real-time reverse transcription-PCR. Total RNA was isolated using TRIzol (Invitrogen), and quantitative real-time reverse transcription-PCR (QRT-PCR) was carried out using iCycler thermocycler (Bio-Rad) and the QuantiTect SYBR green RT-PCR kit (Qiagen). PCR was done at the annealing temperature of 59°C with the following primers for β-actin: 5′-TCCTTCCTGGGCATGGAGTC-3′ for sense and 5′-TTCTGCATCCTGTCGGCAATG-3′ for antisense. For hASH1, the annealing temperature of 60°C with the following primers: 5′-TCCCCCAACTACTCCAACGAC-3′ for sense and 5′-CCCTCCCAACGCCACTG-3′ for antisense; for HES-1, the annealing temperature of 57°C with the following primers: 5′-ACTGATTTTGGATGCTCTGAAGA-3′ for sense and 5′-GTATTAACGCCCTCGCACGT-3′ for antisense; and for Hey1, the annealing temperature of 57°C with the following primers: 5′-AGCCGAGATCCTGCAGATGA-3′ for sense and 5′-GCCGTATGCAGCATTTTCAG-3′ for antisense. The expression levels of the transcripts were calculated using the linear exponential phase of amplification throughout 10 to 35 cycles, and each reaction was normalized using the β-actin transcript internal control.

***In vivo* tumorigenicity.** Athymic 4- to 6-week-old nude mice (nu^+/nu^+) were used for the xenograft experiments. Lung cancer cell lines HCC2429, H460, and A549 (1×10^7 cells) were diluted into 200 μL of PBS and injected s.c. into the right posterior legs of nude mice. When the tumors were palpable, the mice were randomly assigned to control or MRK-003–treated groups. Tumors were measured every 2 days using a caliper. Reported tumor size was calculated using the following formula (24): Tumor Size = Length (L) × Width (W).

Statistical analysis. The size of implanted tumors at different time points following treatment were compared between treated and control groups. Unless specifically stated, statistical inference in comparative experiments both *in vivo* and *in vitro* was obtained using unpaired, two-sided Student's t test. For all determinations, the differences were considered significant when P value is ≤ 0.05.

Results

Notch signaling pathway plays a role in lung cancer. We previously have shown that Notch3 is expressed in about 40% of resected lung cancer (13). However, little is known about the prevalence of other Notch receptors or the Notch ligand Jagged1 in lung cancers. We examined 29 lung cancer cell lines and found that the majority of tumors (24 of 29) expressed Jagged1, with the highest expression in A549, HCC2429, HCC95, H2087, H1993, H1648, and H1435 (Fig. 1*A*). Twelve tumors (41%) expressed the activated form of Notch3 (Notch3IC), consistent with our published data on Notch3 expression in resected tissues (13). Little is known about the frequency of other Notch receptors' expression in lung cancer. In our lung cancer panel, 18 (62%) tumors expressed activated Notch2. On the other hand, the prevalence of Notch1 (14%) and Notch4 (10%) in lung cancer is much lower, suggesting that Notch1 and Notch4 do not have significant roles. Notch receptors signal through transcriptional activation of target genes,

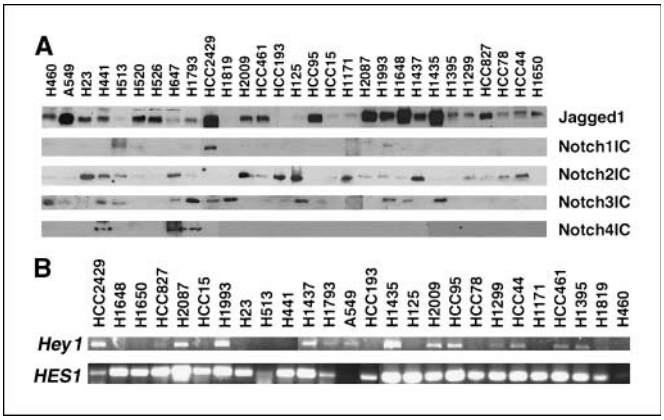

Figure 1. Expression of Notch receptors, ligand, and target genes in lung cancer cell lines. *A,* immunoblots show that Jagged1 is expressed in most lung cancers. In comparison with Notch2 and Notch3 where 18 and 12 of 29 tumors, respectively, express the activated form, activated Notch1 and Notch4 are less frequently observed in lung cancers. *B,* Notch target genes *HES-1* and *Hey-1* are expressed in a majority of tumors, suggesting that the Notch pathway is often altered in lung cancer. Potential relationship between Jagged1, Notch receptors, and target genes were tested using the Fisher's exact test. However, no statistically meaningful relationship was observed, suggesting that there is considerable heterogeneity in the Notch pathway regulation.

Table 1. Expression of selected components of the Notch pathway in lung cancer cell lines

	Jagged1	Notch1	Notch2	Notch3	Notch4	HES1	Hey1
H460	+			+			
A549	+			+			
H23	+		+			+	
H441	+		+	+	+	+	
H513		+	+	+			
H520	+					ND	
H526	+					ND	
H647	+		+	+	+	ND	
H1793	+		+	+	+	+	
HCC2429	+	+		+		+	+
H1819				+		+	
H2009	+		+			+	+
HCC461	+		+			+	+
HCC193			+			+	
H125			+	+		+	
HCC95	+					+	+
HCC15						+	
H1171			+			+	
H2087	+		+			+	+
H1993	+	+	+	+		+	+
H1648	+		+	+		+	
H1437	+		+			+	
H1435	+			+		+	+
H1395	+					+	+
H1299	+		+			+	
HCC827	+		+			+	
HCC78	+		+			+	
HCC44	+		+				+
H1650	+						

NOTE: ND = not analyzed.

HES-1 and Hey1. Consistent with previous reported data using SAGE technology, HES-1 and/or Hey1 are expressed in a significant number of lung cancer cell lines (Fig. 1*B*; ref. 12). Taken together, prevalent expressions of Jagged1, Notch2, Notch3, HES-1, and Hey1 support the hypothesis that the Notch signaling pathway plays an important role in lung cancer. Table 1 summarizes the expression data of selected components of the Notch pathway. We analyzed the data for the association between Notch receptors, ligand, and downstream targets using Fisher exact test. No statistically significant association has been observed, suggesting significant heterogeneity in Notch pathway regulation.

The Notch inhibitor MRK-003 shows antitumor activities *in vitro* and *in vivo*. To determine whether the γ-secretase inhibitor MRK-003 has antitumor effects in human lung cancers, we treated lung cancer cell lines HCC2429, H460, and A549 with increasing doses of MRK-003. The IC_{50} for this compound varies between 5 and 10 μmol/L for HCC2429 and ~25 μmol/L for H460 and A549 for growth on plastic (Fig. 2*A*). Although Lewis et al. (20) observed a time-dependent decrease in cell viability between days 3 and 8 after incubation of T-cell acute leukemia cell lines with MRK-003, there was no change in the IC_{50} observed in lung cancer cell lines at different time points (data not shown), suggesting that the effect of Notch signaling is highly context dependent. *In vivo*, we observed a statistically significant reduction of tumor size in treated xenografts with the exception of A549, where minimal reduction in tumor size is observed (Fig. 2*B*). Histologic examination of HCC2429 tumors resected from xenografts shows marked necrosis in the treated tumor compared with control (Fig. 2*C*). Reduction of activated Notch3 intracellular domain in resected tumor by immunoblotting suggests that the dose of 100 mg/kg given daily for 3 days per week is sufficient to inhibit Notch3 activation and result in tumor reduction (Fig. 2*D*).

Notch inhibition with MRK-003 alters expression of the known Notch effectors and reduces expression of antiapoptotic proteins. Notch receptors are known to signal through binding with CBF1, resulting in the induction of transcription of *HES* (*Hairy enhancer of split*), and HES-related genes. *HES* and related genes are transcriptional repressors. A well-known target of HES proteins is the human homologue of *Achaete-Scute* (*hASH1*). After treatment with MRK-003, transcription of *HES1* and *Hey1*, a HES-related gene, were reduced 4- and 2-fold, respectively. As expected, there is a 4-fold increase in hASH1 transcription (Fig. 3*A*). These observations show that MRK-003 inhibits the canonical Notch signaling pathway. The Notch receptor pathway is known to be important in apoptotic responses (25, 26). To explore the effect of this compound on apoptosis *in vitro*, we examined the Bcl family protein expression after treatment with the inhibitor. We found that MRK-003 reduces pBcl-2 and Bcl-xL expression in a dose-dependent manner in HCC2429. Furthermore, cleaved PARP is

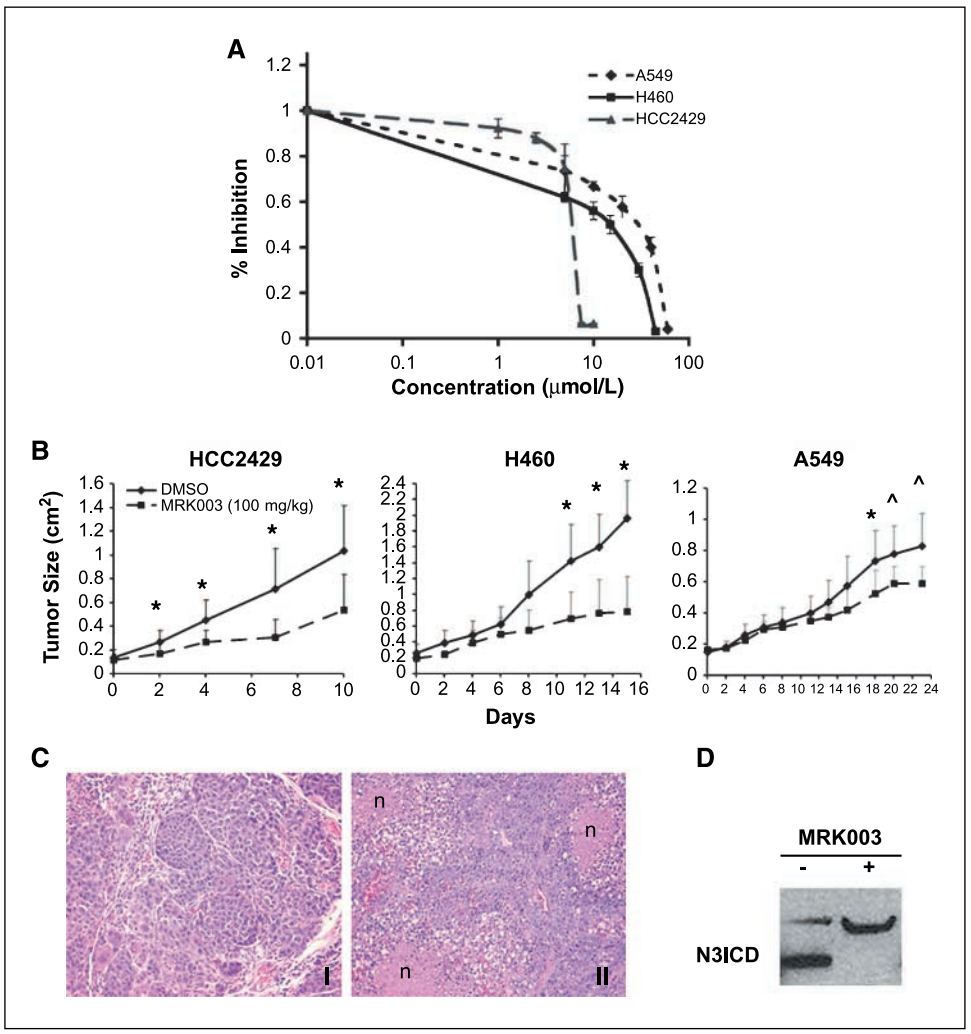

Figure 2. Antitumor effect of the γ-secretase inhibitor MRK-003 on human lung cancer lines expressing activated Notch3. *A,* MRK-003 inhibits growth of lung cancer cells A549, H460, and HCC2429 with varying potency, with HCC2429 being the most sensitive and A549, the most resistant. *B,* the antitumor effect can be observed in lung cancer xenografts. Of note, as observed *in vitro* MTT assay, A549 is similarly least sensitive to γ-secretase inhibition. *, $P \leq 0.05$, statistical significance; ^, $P \leq 0.06$, near statistical significance as calculated using the Student's *t* test. *C,* histologic examination of tumors treated showed marked necrosis in tumor treated in MRK-003. *D,* loss of activated Notch3 ICD is reduced in tumors of treated mice (*lower band*).

increased, suggesting the activation of the caspase pathway (Fig. 3*B, left*). Although expression of cytochrome *c* further indicates that MRK-003 induces apoptosis, no effect on Bax or pAkt levels was seen (Fig. 3*B, right*).

Notch inhibition makes tumors dependent on exogenous growth factors. We have previously shown that Notch3 contributes to growth factor independence in lung cancer cells. To determine whether γ-secretase inhibitors can result in a similar observation, we treated HCC2429 with MRK-003 after 24 h of serum starvation, followed by induction with 10% FCS. No expression of pERK was detected at baseline (0 time) before FCS induction. Induction of pERK can be shown after 15 min of serum stimulation in all conditions. However, after 30 min, a significant reduction of pERK was seen in the treated cells compared with control (Fig. 4*A*). We further showed that MRK-003 significantly enhanced apoptosis in the absence of serum as compared with control. When the cells were maintained in 10% FCS, no change in apoptosis was observed between treated and untreated cells (Fig. 4*B*). The observation that targeting γ-secretase reduces serum independence in cancers suggests that MRK-003 might enhance antitumor activity of growth factor signaling inhibitors, similar to our previous observation when lung cancer cells were treated with a dominant-negative Notch3 receptor (13). To test this hypothesis, we examined the effect of combining MRK-003 with EGFR tyrosine kinase inhibitor, AG1478. Using a soft agar colony assay, we observed a significant decrease in the number of colonies with the combination therapy compared with either treatment alone (Fig. 4*C* and *D*).

Loss of Notch3 in Notch3-overexpressing tumor cell lines results in resistant γ-secretase inhibition. To determine whether the antitumor effect of MRK-003 is dependent on Notch signaling, we created stable transfectants from cell line HCC2429 transfected with plasmid expressing Notch3 siRNA. Of the total seven stable cell lines, clones 5, 6, and 8 (siRNA-C) show persistent Notch3 expression as compared with clones 12, 15, 17, and 20 (siRNA-N3; Fig. 5*C*). The siRNA-N3 clones retained a high level of HES-1, suggesting that HES-1 transcriptional activation is independent of Notch3 activation. Unlike HES1, Hey1 expression is down-regulated, supporting our previous observation that Hey1 is regulated by Notch3 in comparison with HES1 (13). The siRNA-C clones retain sensitivity to inhibition of proliferation by MRK-003 as compared with siRNA-N3 clones (Fig. 5*A*). Resistance to MRK-003–induced apoptosis was also noted in the siRNA-N3 clones as compared with siRNA-C clones (Fig. 5*B*). To further support the hypothesis that MRK-003 antitumor activity is dependent on the retention of Notch3 expression and that the observation were not due to clonal artifacts, we did a similar experiment with transient Notch3 knockdown using siRNA. We showed that the transient loss

of Notch3 in HCC2429 also reduces the effectiveness of MRK-003, particularly after 48 h. These observations further support the hypothesis that the antitumor activity of MRK-003 is Notch dependent.

Discussion

Notch signaling plays a fundamental role in normal development and cell-fate determination in a variety of multicellular organisms. Genes important in normal differentiation often contribute to tumor promotion and survival when they became dysregulated. Although Notch signaling can be growth-promoting or growth-inhibitory depending on cellular context, in NSCLC, its function seems to be oncogenic (12). In our laboratory, we have observed frequent overexpression of HES-1 and/or Hey1 using RT-PCR, suggesting that Notch pathway activation is a frequent event in lung cancer. Given the high mortality of patients with advanced lung cancer, identification of new targets for intervention is crucial for improving these outcomes, and Notch signaling represents one such potential target for intervention.

Involvement of γ-secretase protein complexes in the production of the β-amyloid component of plaques in the brains of patients suffering from Alzheimer's disease has led to the design of inhibitors for these proteases. Because γ-secretase is required for proteolytic cleavage of Notch receptors, we hypothesized that these inhibitors also show activity against Notch signaling in lung cancer cells. Inhibitors of γ-secretase have been shown by some investigators to reduce angiogenesis and induce apoptosis in other systems, further supporting the hypothesis that these compounds may have utility in the treatment of patients with cancer (17–19, 21). Although Notch signaling has been shown to be important in lung cancer biology, to date, the effects of these inhibitors on lung cancer is largely unknown.

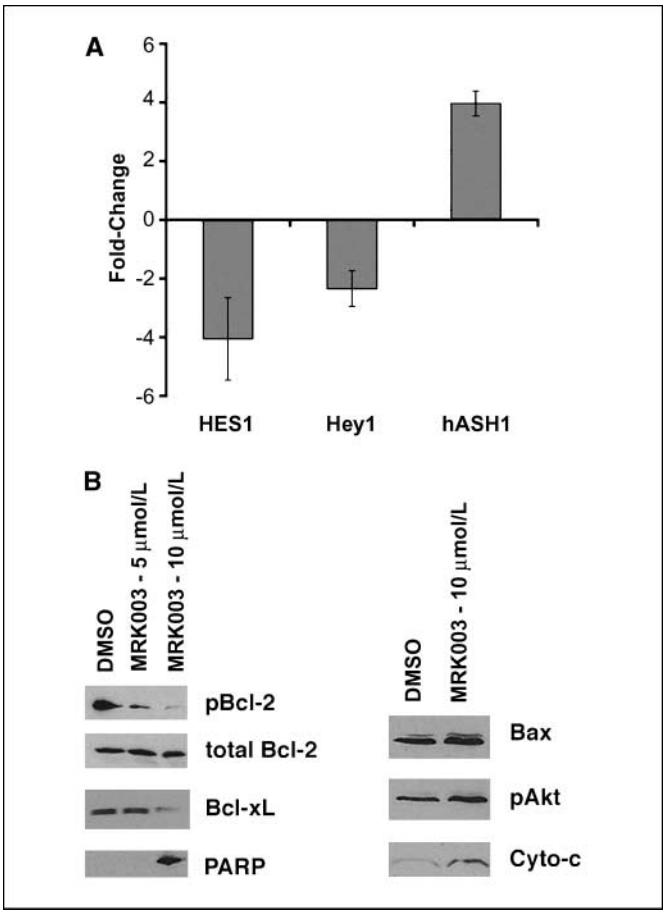

Figure 3. *A,* MRK-003 inhibits transcription of Notch target genes, *HES-1* and *Hey1*. Human homologue of Achaete-Scute (hASH1) is a target of HES-1–mediated transcriptional repression. The loss of HES-1 by MRK-003 results in increased transcription of hASH1. *B,* MRK-003 induces apoptosis through the down-regulation of prosurvival proteins. A profile of prosurvival and proapoptotic proteins shows that MRK-003 induces apoptosis through the down-regulation of prosurvival proteins pBcl-2 and pBcl-xL and not Bax. Interestingly, pAkt was not affected by MRK-003.

In our present study, we showed antitumor activity of the γ-secretase inhibitor MRK-003 in both *in vitro* and in tumorigenicity models. Treatment with MRK-003 resulted in the down-regulation of pMAPK, suggesting one mechanism of biological activity. Induction of PARP and the effects on pBcl-2 and pBcl-xL expression also suggests that the antitumor effect of MRK-003 is also mediated through the effects on apoptosis. This effect on pro-survival proteins is consistent with the growing body of literature supporting the role of Notch signaling in apoptosis, the detailed mechanism of which is largely unknown. However, there are data suggesting that Notch modulates apoptosis through the regulation of nuclear factor-κB (26, 27). Thus, the spectrum of activity we have observed for the γ-secretase inhibitor in lung cancer is consistent with known effects of Notch activation, suggesting that this is the dominant target for this inhibitor in lung cancer.

However, γ-secretase is known to mediate the proteolysis of several transmembrane proteins in addition to Notch receptors, including APP, E-cadherin, CD44, and Erb4 (28–31). Although the loss of activated Notch3 with the concomitant reduction transcriptional expression, Hey1 and HES-1 confirms that MRK-003 targets Notch3 processing; this observation alone does not prove that all of the antitumor effect is Notch3 dependent. However, our studies demonstrating resistance to MRK-003 in Notch3-null clones do support the hypothesis that the dominant antitumor effects observed from MRK-003 are through effects on Notch3 proteolysis in the Notch3-overexpressing cell line HCC2429. Although Notch1 is also expressed in HCC2429, complete abrogation of the MRK-003 effect on apoptosis by Notch3 siRNA suggests that Notch1 do not contribute significantly to the survival of these tumor cells. Further studies are needed to clarify the respective roles of each receptor in lung cancer.

Another approach toward examining the specificity of γ-secretase inhibitors is to determine whether transfection of tumor cells with activated, intracellular domain of Notch3 (N3ICD) can rescue the effect of these inhibitors on proliferation and apoptosis as has been shown in Kaposi's sarcoma for Notch1, 2, and 4 (18). However, the potential nonphysiologic effects of overexpression of an activated transcriptional domain such as the Notch intracellular domain can potentially lead to the potentiation of growth of other physiologic pathways despite drug inhibition. Finally, CD44, E-cadherin, and ErbB-4 have all been identified as potential γ-secretase targets and thus could contribute to the effects we have observed, especially the synergy with EGF TKIs.

In summary, our study shows the antitumor activity of a γ-secretase inhibitor MRK-003 in lung cancer cells. Our data showed that MRK-003 induces apoptosis and reduces tumor growth *in vivo*. The lack of efficacy in lung tumors with knockdown of Notch3

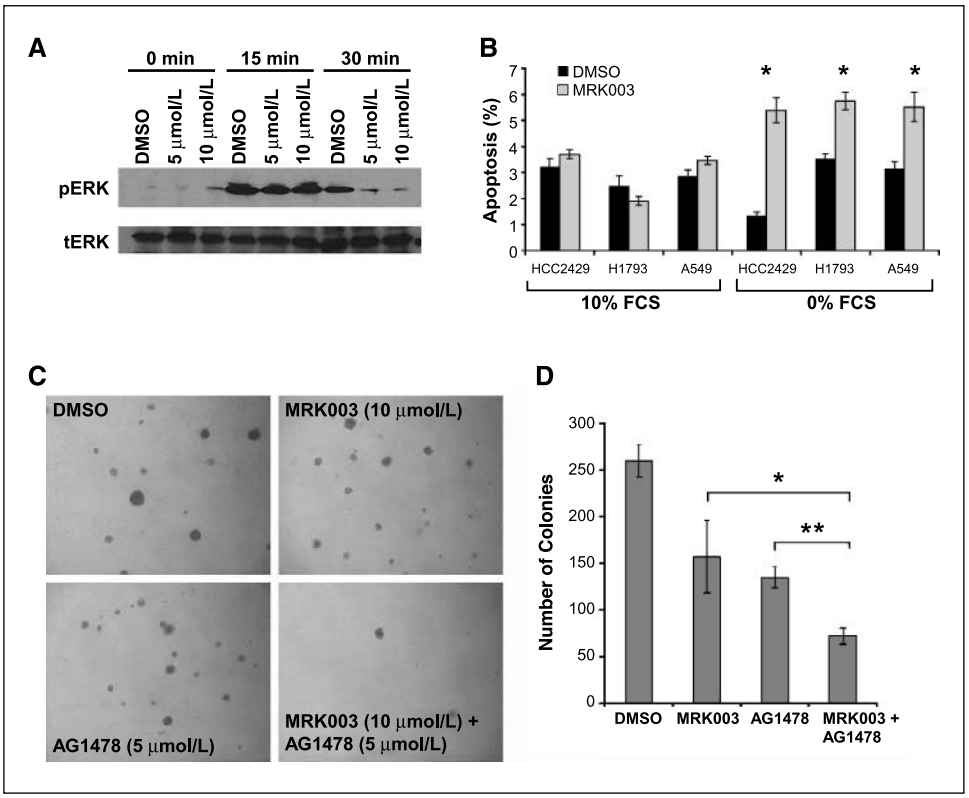

Figure 4. γ-Secretase inhibition results in the down-regulation of pERK and increased dependence in exogenous growth factors. *A,* HCC2429 cells were serum starved overnight and induced with 10% FCS. No change was observed at 0 and 15 min. However, after 30 min following serum induction, treatment with MRK-003 leads to marked reduction of pERK. *B,* significant change in apoptosis as measured by annexin V in treated cells when maintained in serum-free environment. No significant difference is noted in treated samples and control in 10% serum. Notch cooperates with other growth factor pathways in oncogenesis (*C* and *D*). Treatment with MRK-003 reduces colony formation in soft agar, and this reduction is enhanced when combined with AG1478, an inhibitor to EGFR tyrosine kinase. *, $P \leq 0.05$, statistical significance when MRK-003 or AG1478 is compared with DMSO; **, $P \leq 0.05$, statistical significance when compared with single agent MRK-003 or AG1478.

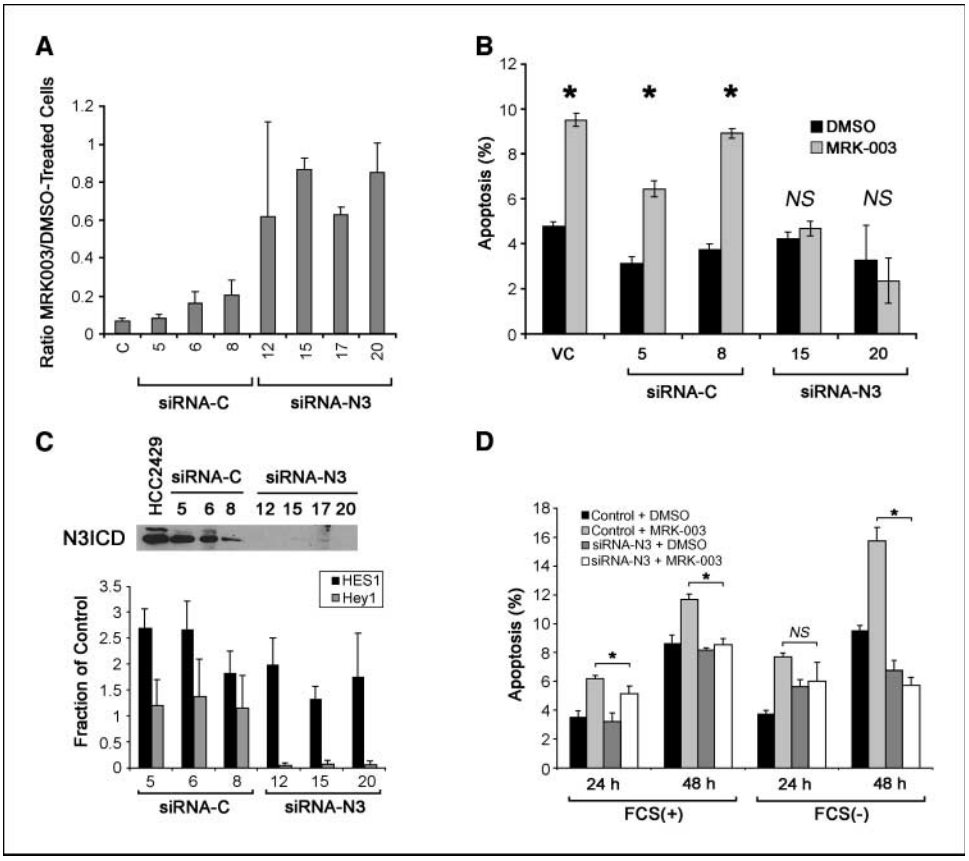

Figure 5. Loss of Notch3 expression renders cancer cells resistant to γ-secretase inhibitor MRK-003. In control HCC2429 and siRNA-C clones 5, 6, and 8 in which the expression of activated Notch3 is retained (*C*), proportions of cells that survived after treatment with MRK-003 to DMSO are markedly reduced as measured by the MTT assay (*A*). Difference in the means between the two groups was statistically significant ($P < 0.05$). *C,* clones retaining Notch expression show higher expression of Hey1, whereas no difference in HES1 is observed between the two groups, suggesting that HES1 is independent of Notch3. In comparison, siRNA-N3 clones 12, 15, 17, and 20, where Notch3 expression is lost, are no longer sensitive to MRK-003. *B,* a similar observation is made in measuring the sensitivity of these cells to MRK-003–induced apoptosis. Induction of apoptosis is also inhibited when Notch3 was transiently knocked down with siRNA in both supplement and serum-free conditions after 48 h (*D*). *, $P \leq 0.05$, statistical significance; *NS*, not statistically significant.

supports our hypothesis that the dominant mechanism of activity is via inhibition of Notch3 activation. Finally, induction of dependency on exogenous growth factors by this compound suggested that combining MRK-003 with other biological compounds can enhance antitumor activity and represents a novel strategy for the targeted therapy of lung cancer.

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