The Gamma Secretase Inhibitor MRK-003 Attenuates Pancreatic Cancer Growth in Preclinical Models

Masamichi Mizuma1, Zeshaan A. Rasheed2, Shinichi Yabuuchi1, Noriyuki Omura1, Nathaniel R. Campbel1, Roeland F. de Wilde1, Elizabeth De Oliveira2, Qing Zhang3, Oscar Puig3, William Matsui2, Manuel Hidalgo2, Anirban Maitra1,2, and N.V. Rajeshkumar2

Abstract
Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy, with most patients facing an adverse clinical outcome. Aberrant Notch pathway activation has been implicated in the initiation and progression of PDAC, specifically the aggressive phenotype of the disease. We used a panel of human PDAC cell lines as well as patient-derived PDAC xenografts to determine whether pharmacologic targeting of Notch pathway could inhibit PDAC growth and potentiate gemcitabine sensitivity. MRK-003, a potent and selective γ-secretase inhibitor, treatment resulted in the downregulation of nuclear Notch1 intracellular domain, inhibition of anchorage-independent growth, and reduction of tumor-initiating cells capable of extensive self-renewal. Pretreatment of PDAC cells with MRK-003 in cell culture significantly inhibited the subsequent engraftment in immunocompromised mice. MRK-003 monotherapy significantly blocked tumor growth in 5 of 9 (56%) PDAC xenografts. A combination of MRK-003 and gemcitabine showed enhanced antitumor effects compared with gemcitabine in 4 of 9 (44%) PDAC xenografts, reduced tumor cell proliferation, and induced both apoptosis and intratumoral necrosis. Gene expression analysis of untreated tumors indicated that upregulation of NF-κB pathway components was predictive of sensitivity to MRK-003, whereas upregulation in B-cell receptor signaling and nuclear factor erythroid-derived 2-like 2 pathway correlated with response to the combination of MRK-003 with gemcitabine. Our findings strengthen the rationale for small-molecule inhibition of Notch signaling as a therapeutic strategy in PDAC.

Mol Cancer Ther; 11(9); 1999–2009. ©2012 AACR.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is one of the most devastating human malignancies characterized by extensive local invasion, early systemic dissemination, and pronounced resistance to chemotherapy and radiotherapy (1). Outcomes for patients with advanced PDAC remain poor with limited clinical benefits seen with currently available therapy. For example, gemcitabine as a systemic agent in the treatment of advanced PDAC results in a median survival of less than 6 months. A recent phase III trial of a combination regimen comprised of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) as first-line therapy has been shown to significantly improve median survival compared with gemcitabine alone in patients with advanced PDAC (2). Nonetheless, the safety profile of FOLFIRINOX was less favorable than that of gemcitabine, and many patients remain ineligible to receive FOLFIRINOX (3). Despite these ongoing advances, the continued poor survival in advanced PDAC underscores the need for new systemic therapies.

Notch signaling is an evolutionarily conserved pathway that plays an important role in multiple cellular and developmental processes (4). Although the Notch pathway plays a pivotal role in normal cell development, aberrant Notch signaling pathway has been extensively linked to a range of human malignancies, rendering this pathway a compelling target for drug development (5, 6). Activation of canonical Notch signaling involves a series of proteolytic processing steps following ligand binding. The interaction of Notch ligands (Jagged and Delta-like) with their receptors promotes γ-secretase–dependent cleavage of the Notch receptor and release of the Notch intracellular domain, which translocates to the nucleus, a requirement for activation of gene targets. Accumulating evidence supports the use of small-molecule inhibitors of
γ-secretase (GSI) as a tractable avenue for Notch antagonism against a number of cancers linked with increased pathway activity (7, 8).

Several lines of preclinical evidence suggest that sustained activation of Notch signaling pathway contributes to the initiation and maintenance of PDAC (9). For example, in primary PDAC tissues, as well as in noninvasive precursor lesions of adenocarcinoma, multiple pathway components, including Notch ligands, receptors, and target genes are overexpressed, relative to normal pancreas (10). In an autochthonous mouse model of PDAC, pharmacologic Notch inhibition attenuates the development of intraductal precursor lesions and invasive cancer, implicating this pathway in tumor initiation (11). Furthermore, downregulation of Notch receptors by RNA interference or exposure to GSI in established human pancreatic cancer cells results in reduced proliferative rates, increased apoptosis, decreased anchorage-independent growth, and decreased invasive properties, suggesting a role for Notch signaling in PDAC maintenance as well (11, 12).

In this study, we assessed the effects of MRK-003, a potent and selective GSI (13, 14), in preclinical models of PDAC. MRK-003 is the preclinical analog of MK-0752, a GSI that is currently in clinical development (15, 16). We used a panel of human PDAC cell lines, as well as patient-derived PDAC xenografts, to determine whether pharmacologic targeting of the Notch pathway using MRK-003 could curb tumor growth and potentiate gemcitabine sensitivity. Herein, we also attempt to delineate a gene signature of responsiveness to Notch inhibition, which may aid in the selection of patient subsets more likely to benefit from this strategy in the clinic.

Materials and Methods

Drugs

MRK-003 was provided by Merck Research Laboratories. Gemcitabine (Eli Lilly) was purchased from the Johns Hopkins Hospital Pharmacy. Structures of MRK-003 and gemcitabine are shown in Fig. 1 (17, 18).

Figure 1. Chemical structures of MRK-003 and gemcitabine. MRK-003 (A) and gemcitabine (B).

Cell lines

Authenticated low-passage PDAC lines (Pa03C, Pa14C, Pa16C, and Pa29C), established at Johns Hopkins, were used for the study. Culture conditions and exome-wide somatic mutational status of these cell lines has been previously described (19). In addition, we used Capan-1 [American Type Culture Collection (ATCC)] on the basis of its Notch dependence (12). Cell line provider (ATCC) used cytochrome c oxidase I PCR assay, short tandem repeat profiling, and Ouchterlony diffusion method to characterize the cell line.

Quantitative real-time reverse transcription PCR

Cells were collected after incubation with MRK-003 (2 or 5 μmol/L) for 48 hours. Total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was synthesized with SuperScript First Strand System (Invitrogen). qRT-PCR was conducted using FAST SYBR Green Master Mix (Applied Biosystems) on a Step One Plus Real-Time PCR System (Applied Biosystems). Human PGK1 and murine β-actin were used as housekeeping genes. Relative expression of the mRNA was estimated using the 2^ΔΔCt method (20).

Anchorage-independent growth

Anchorage-independent growth of cells was determined by soft agar assays in 6-well plates. Briefly, cells were incubated in media containing 0.5% FBS with vehicle or MRK-003 (2 or 5 μmol/L). After incubation for 48 hours, the treated cells were recovered by media with 10% FBS for 24 hours. Thereafter, equal numbers of viable cells from each condition were quantified using a hemocytometer with trypan blue counterstain, and then plated for soft agar assays. A bottom layer of 1% agarose, a middle layer of 0.6% agarose including 10,000 cells and a top layer of medium only were applied into each well. After incubating the plates for 3 weeks, colonies were stained with crystal violet solution, visualized by trans-UV illumination and counted using the analysis software Quantity One (BioRad).

Stable overexpression of Notch1 intracellular domain

Stable transfectants overexpressing the Notch1 intracellular domain (N1ICD) was established in Pa03C cells, as previously described (12). The stable transfectants were maintained in media supplemented with 600 μg/mL of G418. Mock vector was transfected as a control. Overexpression of N1ICD compared with empty vector–transfected cells was confirmed by qRT-PCR (12).

Protein extraction and Western blotting

Both N1ICD stable transfected as well as empty vector–transfected Pa03C cells were cultured separately in tissue culture flasks. Cells were trypsinized and cell pellets were lysed using lysis buffer. Western blots were conducted as previously described (21). Membranes were incubated with primary antibodies against rabbit N1ICD (Val1744)
and Hes-1 (Cell Signaling Technology, Inc. and Abcam, respectively). Membranes were probed with secondary HRP-conjugated antibody (GE Healthcare) and bound antibodies were detected by SuperSignal West Pico/ Femto chemiluminescent substrate (Thermo Scientific). Equal loading was verified with β-actin antibody.

**Engraftment of ex vivo pretreated PDAC cells in athymic mice**

Male athymic nude mice (6-week-old, Harlan) were housed and maintained in accordance with the Institutional Animal Care and Use Committee and guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. PDAC cells were treated ex vivo with either vehicle or with MRK-003 (5 μmol/L) for 48 hours, followed by a recovery in full serum conditions for an additional 24 hours, before subcutaneous injection. Viable 5 × 10⁶ cells in a total volume of 200 μL of 1:1 (v/v) PBS/Matrigel (BD Biosciences) were injected subcutaneously into bilateral flanks (right flank, cells pretreated with vehicle; left flank, cells pretreated with MRK-003) of mice (N = 6). Tumor size was measured with digital calipers.

**FACS analysis of tumor-initiating cells (CD44⁺/CD24⁻ and ALDH¹ cancer cells)**

PDAC cells were treated with MRK-003 (2 or 5 μmol/L) for 48 hours. The cells were harvested and stained with ALDEFLUOR. Briefly, 1 million cells were resuspended in 1 mL ALDEFLUOR buffer and 1 μL ALDEFLUOR reagent in the presence or absence of the ALDH1 inhibitor, diethylamino-benzaldehyde (DEAB), for 30 minutes in a 37°C water bath. The cells were washed and incubated at 4°C for 15 minutes with monoclonal anti-CD44-allophycocyanin (APC) (1:20 dilution; clone G4-26, BD Biosciences) and anti-CD24-phycocerythrin (PE) (1:20 dilution; clone ML5, BD Biosciences) antibodies. The cells were washed and resuspended in ALDEFLUOR buffer containing 2 μg/mL propidium iodide (PI). A FACSCalibur flow cytometer (BD Biosciences) was used for flow cytometric analysis, as previously described (22). The cells were first gated on the basis of side-scatter and forward-scatter properties, followed by exclusion of nonviable (PI-positive) cells. The ALDH¹+ gate was created on the basis of DEAB-treated cells stained with ALDEFLUOR, anti-CD24-PE, and anti-CD44-APC. The CD44⁺/CD24⁻ gates were created on the basis of cells stained with ALDEFLUOR, mouse-specific IgG2b k-APC (1:100 dilution; BD Biosciences) and IgG2a k-PE (1:100 dilution; BD Biosciences) antibodies (22, 23). Gates were created on the basis of cellular staining with isotype control antibodies. FACS plots for these controls are shown as Supplementary Fig. S1.

**Notch1 gene expression**

RNA isolated from baseline (untreated) tumors of 30 individual patient-derived pancreatic cancer xenografts was profiled using Affymetrix U133 Plus 2.0 gene arrays. Sample preparation and processing procedure was conducted as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc.). Gene expression levels were converted to a rank-based matrix and standardized for each microarray (23).

**Xenograft establishment and in vivo efficacy studies**

Low-passage PDAC xenografts, established at Johns Hopkins Hospital, were used for the study (24). Nine independent patient-derived xenografts were selected for the in vivo efficacy study, based on elevated Notch1 expression. Cohorts of athymic mice with tumor size of approximately 200 mm³ were randomized to 4 arms (5 mice; 10 tumors per group): (i) vehicle control, (ii) MRK-003 150 mg/kg p.o. once weekly for 3 weeks, (iii) gemcitabine 30 mg/kg i.p. once weekly, for 3 weeks, and (iv) combination of MRK-003 and gemcitabine in the above mentioned dose and schedule. Tumor size was evaluated twice weekly and tumor volume was calculated using the following formula: tumor volume = (length × width²)/2.

**Histology, immunohistochemistry, and fluorescence microscopy**

Excised tumors were fixed in 10% buffered formalin before paraffin embedding. Immunohistochemical staining of nuclear anti-Notch1 (Abcam; ab8925, 1:400 dilutions) and nuclear anti-Hes1 (Abcam; ab49170, 1:250 dilutions) were conducted as per the manufacturer’s protocol. Immunohistochemistry for proliferation (Ki-67 antigen) was conducted using an anti-MIB-1 (Ki-67) antibody (clone K2, dilution 1:100, Ventana Medical Systems) as previously described (25). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was done by a commercial apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega), according to the recommendations of the manufacturer (25). We used Vectashield with 4′,6-diamidino-2-phenylindole to preserve fluorescence and to counterstain nuclei. Sections were examined microscopically and the average number of cells stained positive for Ki-67 and TUNEL were counted in 5 random fields each from the tumors of 2 separate animals under at ×20 magnification.

**Gene expression analysis**

RNA isolated from the 9 parental (untreated) PDAC xenografts was profiled using Affymetrix U133 Plus 2.0 gene arrays at least in duplicates. This gene array has approximately 54,000 probes comprising approximately 20,000 RefSeq genes. CEL files generated from the array were normalized using the robust multiarray average algorithm (26). Pearson correlation coefficients were calculated to identify genes that show significant correlation (P < 0.01) with tumor growth inhibition upon drug treatment. Pathway and biologic function analysis of gene sets was conducted using Ingenuity Pathway Analysis (IPA, Ingenuity Pathway Analysis), and overlap of gene sets with other publicly available gene signatures was
examined using NextBio (NextBio). Microarray raw data are available in GEO (Accession number: GSE37645).

Statistical analysis

All groups were studied in parallel and differences between groups were analyzed by ANOVA, as appropriate, and Bonferroni multiple comparison tests conducted using GraphPad Prism. The difference between experimental groups was considered significant when the P value was <0.05. Bar and line graphs show mean ± SEM, respectively if not otherwise indicated.

Results

MRK-003 treatment downregulates the expression of Hes-1 in PDAC cells

To determine whether MRK-003 could modulate Notch target genes, we examined the expression of Hes-1 transcripts in a panel of 5 PDAC cell lines (Capan-1, Pa03C, Pa14C, Pa16C, and Pa29C). Hes-1 transcripts were down-regulated in all pancreatic cancer cell lines treated with both 2 and 5 μmol/L of MRK-003 (Fig. 2A).

MRK-003 pretreatment inhibits anchorage-independent growth

Anchorage-independent growth in soft agar was significantly reduced upon exposure to MRK-003 in Capan-1 at both 2 and 5 μmol/L dosages (P < 0.01), whereas significant inhibition in the other 2 "sensitive" lines (Pa03C and Pa14C) was observed at 5 μmol/L dosing (Fig. 2B). In contrast, the remaining 2 PDAC lines—Pa16C and Pa29C—were resistant to MRK-003 at both 2 and 5 μmol/L dosages (Fig. 2C), despite the downregulation of Hes-1 transcripts observed previously, suggesting that other Notch effectors were involved in this phenotype.

Enforced expression of N1ICD rescues in vitro effects of MRK-003

To determine if enforced Notch expression could rescue the MRK-003 phenotype, stable transfectant of...
N1ICD and mock vectors were established in Pa03C cells (Pa03C-N1ICD and Pa03C-mock, respectively). Stable expression of N1ICD was confirmed by upregulation of transcript corresponding to the ICD portion of NOTCH1, as well as the overexpression of N1ICD and Hes-1 protein compared with the mock-transfected Pa03C cells (Fig. 2D and E). Enforced N1ICD expression per se augmented colony formation significantly compared with the Pa03C-mock clones in vehicle-treated cells (Fig. 2F, \( P < 0.001 \)). In Pa03C-mock cells, treatment with MRK-003 at 5 \( \mu \)mol/L reduced colony numbers by approximately 50% (\( P < 0.001 \)), whereas in Pa03C-N1ICD cells, equimolar MRK-003 attenuated colony formation by approximately 10%, which was not statistically significant (Fig. 2G).

**MRK-003 pretreatment delayed tumor engraftment**

To determine whether pretreatment with MRK-003 could modulate tumorigenicity in mice, we monitored the tumor growth of subcutaneously implanted Capan-1 and Pa03C cells, which had been pretreated \( \text{ex vivo} \) with either MRK-003 or vehicle. Engraftment was significantly delayed at day 14 and day 21 postimplantation in both cell lines (Fig. 3A and B). In contrast, there was no difference in engraftment rates for either Pa16C or Pa29C cells, between the control and MRK-003 pretreatment arms (Fig. 3B). The differential sensitivity to MRK-003 was consistent with the prior data in anchorage-independent growth obtained using this panel of cells.

**Effect of MRK-003 treatment on tumor-initiating cells**

We examined the effect of MRK-003 treatment on 2 partially overlapping populations of cells, CD44\(^{+}\)CD24\(^{+}\) and ALDH\(^{+}\), which have independently been associated with tumor-initiating properties in PDAC (22, 27). MRK-003 treatment in Capan-1 resulted in the reduction of CD44\(^{+}\)CD24\(^{+}\) tumor cells at 2 \( \mu \)mol/L, and elimination of these cells at 5 \( \mu \)mol/L (Fig. 4A and B). A comparable observation was noticed in the sensitive Pa03C cell line (Fig. 4B). Similarly, MRK-003 treatment reduced the proportion of ALDH\(^{+}\) cells in Capan-1 and in Pa03C (Fig. 4A and C). However, MRK-003 treatment increased the relative proportion of CD44\(^{+}\)CD24\(^{+}\) and ALDH\(^{+}\) cells in resistant lines (Pa16C and Pa29C), respectively (Fig. 4B and C).

**Effect of MRK-003 treatment on tumor growth, Notch targets, proliferation, and apoptosis**

Thirty patient-derived PDAC xenografts were mined \( \text{in silico} \) for relative expression of NOTCH1 transcripts, using microarray data (Fig. 5A). On the basis of the elevated NOTCH1 expression in the baseline xenografts (relative expression is illustrated as red to green in order of decreasing values), we selected 9 individual patient-derived xenografts for further expansion in mice, and randomization to MRK-003 or gemcitabine monotherapy, or the combination of MRK-003 and gemcitabine. As shown in Fig. 5B and C, MRK-003 monotherapy significantly reduced tumor volume in 5 of 9 xenografts (56%) as...
compared with control, which were Panc374 (P < 0.05), Panc219 (P < 0.05), Panc265 (P < 0.001), Panc420 (P < 0.01), and JH033 (P < 0.01). Moreover, there was a significant decrease in tumor growth in 4 of 9 (44%) xenografts in the combination therapy group as compared with the single-agent gemcitabine, and this subset of tumors was comprised of Panc198 (P < 0.05), Panc219 (P < 0.01), Panc291 (P < 0.001), and JH033 (P < 0.05; Fig. 5C). Baseline Notch1 expression (Fig. 5A) was not predictive of GSI (MRK-003) sensitivity in this tumor model.

MRK-003 as well as combination of gemcitabine with MRK-003 treatments reduced the expression of both nuclear Notch1 (N1ICD) and Hes-1 as compared with single-agent therapy, xenografts receiving MRK-003 and gemcitabine showed significantly increased apoptosis (as assessed by TUNEL staining; Fig. 7A), as well as significantly reduced tumor cell proliferation (as assessed by Ki-67; Fig. 7B).

Gene expression analysis for predictive signatures of response to MRK-003

Probe sets that show significant correlation with response to MRK-003, or the combination of MRK-003 plus gemcitabine (692 and 967 probes, respectively, Supplementary Tables S1 and S2) were identified with Pearson r value >0.1, and subjected to IPA, as well as overlap with publicly available expression datasets using NextBio. IPA showed that upregulation of NF-κB signaling function in the parental xenografts correlated with response to MRK-003 in vivo (Supplementary Fig. S2A).

NextBio analysis of MRK-003 sensitivity associated probes showed that 75% of the single-agent response signature in PDAC xenografts overlapped with that of trimethylated histone H3 (H3K4me3) bound genes (28) with high significance (overlap P value = 8.2E-53). We then assessed for potential signatures of response to the combination of MRK-003 and gemcitabine using the IPA platform. Upregulation of B-cell receptor (BCR) signaling (Supplementary Fig. S2B), as well as the nuclear factor erythroid-derived 2-like 2 (NRF2)-mediated oxidative stress response pathways (data not shown) were significantly associated with the sensitivity of MRK-003 plus gemcitabine combination. NextBio analysis of the combination response signature showed that, in addition to the previously observed overlap with the H3K4me bound genes, 9% of the response signature also overlapped with NRF1 binding site gene set from the Broad MsigDB—Regulatory Motifs (overlap P value = 2.6E-17), in agreement with IPA’s association with NRF2-mediated canonical signaling pathway. Of interest, there was a negative correlation between the combination response signature with the signature attributable to rituximab (anti-CD20 antibody) response, which is in agreement with the positive association with BCR signaling detected by IPA, as rituximab specifically inhibits this signaling axis (29).

Discussion

Given its well-defined role in PDAC growth, Notch signaling has been an area of intense investigation (30, 31). A recent exomic profiling of PDAC has identified Notch as 1 of the 12 core signaling pathways that are abnormal in this neoplasm (19). Notch pathway components are enriched in PDAC patients resistant to

Figure 4. MRK-003 treatment modulates the population of tumor-initiating cells in PDAC lines. PDAC cells were treated with MRK-003 (2 or 5 µmol/L) for 48 hours. The cells were harvested and stained for FACS analysis. A, representative data for CD44+CD24+ and ALDH+ population in MRK-003 treated in Capan-1 cells showing reduction of CD44+CD24+ and ALDH+ cells compared with the vehicle-treated cells. B and C, percentage of CD44+CD24+ and ALDH+ population, respectively, in 4 pancreatic cancer cell lines treated with MRK-003 for 48 hours. Dose-dependent reduction in the proportion of CD44+CD24+ and ALDH+ cells were noticed in 2 sensitive cell lines (Capan-1 and Pa03C). However, MRK-003 increased the proportion of CD44+CD24+ and ALDH+ cells in Pa16C and Pa29C. Experiments were conducted in triplicate, N = 3, error bars SD.
gemcitabine treatment and other molecular targeted agents (24, 32). The overwhelming majority of PDAC harbor activating mutations of KRAS (19), and sustained Notch1 signaling was shown to be essential in maintaining the transformed phenotype of Ras-mutant cells (33). These multiple lines of evidence strongly support the pharmacologic targeting of Notch signaling in PDAC, particularly in combination with gemcitabine.

MRK-003 is a potent and selective GSI (13, 14), which is the preclinical analog of MK-0752, currently in clinical development (15, 16). As a "pan" Notch inhibitor, MRK-003 is expected to inhibit the enzymatic cleavage of all 4 Notch receptors, which bypasses the need for receptor-specific targeting based on tumor context. For example, our studies have shown that Notch1 is the primary oncogenic influence in pancreatic cancer (10), whereas lung...
cancers harbor greater dependence on Notch3 signaling (13), and medulloblastomas on Notch2 (34). In the context of breast cancer, activation of Notch1 is observed in tumors exposed to trastuzumab, contributing to therapeutic resistance (35). On the contrary, combining MRK-003 with trastuzumab completely prevented tumor recurrence in trastuzumab-sensitive tumor, whereas the combination of lapatinib with MRK-003 significantly reduced tumor growth in trastuzumab-resistant cancers (36).

Our *in vitro* results confirm that MRK-003 exposure leads to differential effects in PDAC cells, with a subset of lines unequivocally responding to the agent by significant reduction in anchorage-independent growth and delay in tumor engraftment in athymic mice; in contrast, other lines remain refractory to the drug. Although MRK-003 treatment could diminish the proportion of CD24+ CD44+ or ALDH+ subpopulation in Capan-1 and Pa03C cells, the treatment leads to the enrichment of CD24+ CD44+ and ALDH+ cells in Pa16C and Pa29C cells. Although our current study was entirely geared toward the use of MRK-003 as a therapeutic strategy in established PDAC, Plentz and colleagues have previously shown the significant efficacy of MRK-003 in chemoprevention using a genetically engineered mouse model (11), further underscoring the importance of Notch signaling to tumor initiation in the pancreas. It is important to note that the cellular phenotypes implicated in tumor initiation are also highly enriched in pancreatic cancer metastases (22, 37), and thus, pharmacologic Notch inhibition would conceivably be beneficial in advanced disease settings as well.

A recent report indicated that a combination of MRK-003 and gemcitabine could induce intratumoral necrosis in KPC mouse model (38). In light of this, we examined the histologic sections from treated xenografts and find that stroma-rich xenografts (e.g., Panc374; Supplementary Fig. S3) do, in fact, show increased necrosis in the combination therapy arm. Although central necrosis to some degree is observed in all of the cohorts, the MRK-003 plus gemcitabine-treated tumors show sheets of confluent necrosis. However, this phenomenon was not uniformly observed, especially in stroma-poor xenografts (data not shown). We believe that the relative hypovascularity of stroma-rich xenografts (a feature also reflected in human pancreatic cancers and the KPC model) may explain why...
these tumors show increased necrosis in the setting of combination therapy.

As an attempt to identify predictive signatures in response to MRK-003, we examined the baseline gene expression profiles of the 9 patient-derived xenografts as it related to variable \textit{in vivo} responses to either MRK-003 alone or the combination regimen. Upregulation of NF-\(k\)B signaling components was correlated with response to single-agent MRK-003, a finding that attains significance in light of the recent observation of cross-talk between Notch and NF-\(k\)B signaling in murine PDAC models (30). In that study, NF-\(k\)B activation collaborated with basal Notch signals to enhance Notch target gene expression, suggesting that a signature consistent with NF-\(k\)B activation may indicate a greater degree of Notch dependence in PDAC cells. The existence of crosstalk between Notch and NF-\(k\)B pathways has also been reported by other groups using \textit{in vitro} models of pancreatic cancer (39, 40), underscoring the validity of our findings. In terms of the combination

Figure 7. Combination of gemcitabine and MRK-003 induces apoptosis and reduces cell proliferation in PDAC xenografts. A, representative photomicrograph of TUNEL staining from Panc198 and Panc291. Histogram of TUNEL-positive nuclei per high-power field (bottom), showing that combination of gemcitabine and MRK-003 significantly enhanced apoptotic cells per field as compared with the gemcitabine treatment. B, representative photomicrograph of Ki-67 staining from Panc198 and Panc291. Histogram of Ki-67-positive nuclei per high-power field (bottom), showing that combination of gemcitabine and MRK-003 significantly reduced proliferating cells as compared with the gemcitabine treatment. Histograms for TUNEL and Ki-67 were generated by evaluating 5 high-power fields per xenograft section from 2 independent tumors per treatment arms; mean \(\pm\) SEM (*, \(P < 0.001\) compared with the gemcitabine). GEM, gemcitabine.
regimen, we identified 2 activation signatures that correlated with response in xenografts, BCR signaling and Nrf2, respectively. Although an interaction between BCR and Notch signaling has not been reported previously in solid tumors, not unexpectedly a potential synergy does exist in normal and neoplastic hematopoietic cells (41, 42). Thus, in both normal B-cells, as well as in B-cell lymphoma lines, Notch promotes BCR-mediated proliferation, which is blocked by administration of GSI (41, 42). In these aforementioned studies, putative downstream effectors that have been implicated in the observed synergy include the MAP kinase signaling pathway and Myc, both of which retain considerable significance in PDAC pathogenesis. The correlation between sensitivity to GSI plus gemcitabine and Nrf1/2 signaling (which was identified by 2 disparate strategies, IPA and NextBio analyses, respectively) is, to the best of our knowledge, previously unreported. As recently showed, Nrf2 expression is crucial to most oncogene-driven cancers (including PDAC), in light of its ability to attenuate deleterious reactive oxygen species that accumulate from oncogene-induced cellular stress (43). The precise cellular mechanisms by which an Nrf2 activation signature leads to sensitivity to MRK-033/gemcitabine regimen remain to be determined; nonetheless, the in silico correlations generated with regard to various activation signatures (NF-xB, BCR, and Nrf2) in PDAC and response to either single-agent GSI or combination regimens should be testable hypotheses, as these agents are increasingly being evaluated in the clinic.

Substantial preclinical evidence supports the role of Notch signaling in the initiation, progression, and maintenance of PDAC. Pharmacologic targeting of Notch signaling in PDAC patients using GSI such as MK-0752 is currently being evaluated in advanced PDAC patients (ClinicalTrials.gov, NCT ID: NCT01098344; 44). Our results strengthen the rationale for small-molecule inhibition of γ-secretase inhibition as a therapeutic strategy in PDAC and identification of a putative sensitivity signature to the combination of gemcitabine and MRK-003 may provide insights into clinical response in the ongoing and future trials.

Disclosure of Potential Conflicts of Interest
Q. Zhang and O. Puig are employees of Merck Research Laboratories.

Authors' Contributions
Conception and design: M. Mizuma, Z.A. Rasheed, E. De Oliveira, A. Maitra, N.V. Rajeshkumar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Mizuma, Z.A. Rasheed, S. Yabuuchi, N.R. Campbell, R.F. de Wilde, E. De Oliveira, N.V. Rajeshkumar
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): M. Mizuma, Z.A. Rasheed, S. Yabuuchi, R.F. de Wilde, Q. Zhang, O. Puig, W. Matsuji, A. Maitra, N.V. Rajeshkumar
Writing, review, and/or revision of the manuscript: Z.A. Rasheed, R.F. de Wilde, Q. Zhang, O. Puig, M. Hidalgo, A. Maitra, N.V. Rajeshkumar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Mizuma, N. Omura, E. De Oliveira, Q. Zhang
Study supervision: N.V. Rajeshkumar

Acknowledgments
The authors thank Shweta G. Pai (Johns Hopkins) for help in tissue culture experiments.

Grant Support
N.V. Rajeshkumar, A. Maitra, and M. Hidalgo are supported by a Stand Up To Cancer Dream Team Translational Cancer Research Grant, a Program of the Entertainment Industry Foundation (SU2C-AACR-DT0509). A. Maitra is supported by Merck Research Laboratories and NIH CA113669. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 10, 2012; revised May 23, 2012; accepted June 15, 2012; published OnlineFirst July 2, 2012.

References
Notch Inhibition in Pancreatic Cancer


Molecular Cancer Therapeutics

The Gamma Secretase Inhibitor MRK-003 Attenuates Pancreatic Cancer Growth in Preclinical Models


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0017

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/07/02/1535-7163.MCT-12-0017.DC1

Cited articles
This article cites 43 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/11/9/1999.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/11/9/1999.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.