The MEK1/2 Inhibitor Pimasertib Enhances Gemcitabine Efficacy in Pancreatic Cancer Models by Altering Ribonucleotide Reductase Subunit-1 (RRM1)

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Abstract

Purpose: Gemcitabine, a nucleoside analogue, is an important treatment for locally advanced and metastatic pancreatic ductal adenocarcinoma (PDAC) but provides only modest survival benefit. Targeting downstream effectors of the RAS/ERK signaling pathway by direct inhibition of MEK1/2 proteins is a promising therapeutic strategy, as aberrant activation of this pathway occurs frequently in PDAC. In this study, the ability of pimasertib, a selective allosteric MEK1/2 inhibitor, to enhance gemcitabine efficacy was tested and the molecular mechanism of their interaction was investigated.

Experimental Design: Cell survival and apoptosis were assessed by MTT and Caspase 3/7 Glo assays in human pancreatic cancer cell lines. Protein expression was detected by immunoblotting. The in vivo sensitivity of gemcitabine with pimasertib was evaluated in an orthotopic model of pancreatic tumor.

Results: Synergistic activity was observed when gemcitabine was combined sequentially with pimasertib, in human pancreatic cancer cells. In particular, pimasertib reduced ribonucleotide reductase subunit 1 (RRM1) protein, and this was associated with sensitivity to gemcitabine. Pretreatment with MG132 impaired reduction of RRM1 protein induced by pimasertib, suggesting that RRM1 is degraded posttranslationally. Immunoprecipitation indicated enhanced MDM2-mediated polyubiquitination of RRM1 through Lys-48–mediated linkage following pimasertib treatment, an effect mediated, in part, by AKT. Finally, the combination treatment with pimasertib and gemcitabine caused significant tumor growth delays in an orthotopic pancreatic cancer model, with RRM1 downregulation in pimasertib-treated mice.

Conclusions: These results confirm an important role of RRM1 in gemcitabine response and indicate MEK as a potential target to sensitize gemcitabine therapy for PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease associated with poor prognosis, with few patients surviving 1 year after diagnosis (1). Gemcitabine, an antimetabolite with similar structure to the nucleoside cytidine, (Gem), (2′,2′-difuorodeoxycytidine: dFdC) is an important chemotherapeutic agent for the treatment of locally advanced and metastatic PDAC, although with benefit for only 25% of patients and with median survival of less than 6 months (2).

Gemcitabine is a prodrg, which is actively transported inside the cells through human equilibrative nucleotide transporters (hENT) and transformed into its active form, 2′,2′-difuorodeoxycytidine triphosphate (dFdCTP), by the enzyme deoxycytidine kinase (dCK). Ultimately, gemcitabine gets inactivated through deamination of cytidine and deoxycytidine to form uridine and deoxyuridine by cytidine deaminase (CDA; ref. 3). The main mechanism of action of dFdCTP is the inhibition of DNA synthesis: dFdCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, leading to termination of DNA duplication (6). In addition, gemcitabine binds and irreversibly inactivates ribonucleotide reductase (RNR) large subunit one (RRM1; refs. 7, 8). RNR is the rate-limiting step of DNA duplication (9), as this enzyme exclusively catalyses the
conversion of ribonucleotides into 2'-deoxyribonucleotides required for new DNA synthesis and repair (10). RNR consists of two homodimers, each of them composed of two subunits: a large regulatory subunit RRM1 and a small catalytic subunit RRM2 (11). RRM1 can also bind to PS3R2, a subunit dependent on PS3 activity (9). The two subunits associate, forming an active holoenzyme (11). Levels of RRM1 protein are constant during the cell cycle; RRM2 levels oscillate during the cell cycle with a peak in S-phase (12).

From in vitro and clinical studies, conflicting results have emerged about the biologic role of RRM1 in cancer. RRM1 can act as a tumor suppressor gene: its overexpression reduced cell migration and metastasis in Ras-transformed fibroblasts (13). Furthermore, high expression of RRM1 suppressed formation of metastasis by inducing the expression of tumor suppressor gene PTEN and prolonged survival in a mouse model of lung cancer (14).

In contrast, other in vitro and clinical studies have demonstrated that RRM1 overexpression is associated with gemcitabine resistance in patients with non–small cell lung cancer (NSCLC) and PDAC, with high levels of RRM1 expression linked to worse overall survival (15, 16).

The mitogen-activated protein kinase (MEK)/extracellular signal–regulated kinase (ERK) signal transduction pathway plays a central role in cell proliferation and survival, and it is activated by ligands such as EGF and TGF-β that initiate a cascade of phosphorylating events. The binding of ligands leads to receptor dimerization and stimulation of intracellular RAS, which exchanges GDP for GTP, leading to its activation. Activated RAS phosphorylates and activate RAF protein. Downstream of RAF are MEK or MAPK proteins, dual specificity kinases (17). There are seven MEK proteins identified involved in four different MAPK signaling pathways; only MEK1 and MEK2 belong to the RAS/RAF/ERK signaling pathway. Activation of MEK1 and MEK2 by RAF occurs through phosphorylation of two serine residues at positions 217 and 221. MEK phosphorylates the threonine and tyrosine residues in the activation loop of downstream protein ERK (18). Activated ERK translocates into the cytoplasm and phosphorylates cytoplasmic phosphatases and kinases or activates transcription factors within the nucleus that regulate cell proliferation, survival, as well as angiogenesis and migration (19).

The RAS/RAF/MEK/ERK signaling pathway is frequently deregulated in human cancers. In particular, mutations of the KRAS gene occur in almost 90% of PDAC and have been found in early precursor lesions of PDAC, pancreatic intraepithelial neoplasia (PanIN), where they play an important role in their maintenance and progression (1). MEK is a downstream target of RAS/RAF proteins; therefore, it represents an important target for therapeutic intervention in cancers characterized by the presence of mutated KRAS or BRAF genes (20). Several MEK inhibitors have been developed and are under investigation in preclinical and clinical studies. The most recently developed MEK inhibitors have improved kinase specificity preventing undesired effects arising from off-target interactions. In addition, MEK pathway inhibitors are specific with the only catalytic substrate of MEK represented by ERK (21). Pimasertib (MSC1936369B/AS703026) is a highly selective, small-molecule inhibitor of the protein kinase MEK1 and MEK2 and binds an allosteric site adjacent to the ATP binding site of MEK. Preclinical studies showed that pimasertib induced significant antitumor activity in xenograft models of mutant KRAS colorectal cancer (22). Furthermore, pimasertib treatment induced cell death in multiple myeloma cell lines, patient cells, and xenograft models and enhanced the cytotoxic effect of conventional anti–multiple myeloma therapies (23).

In this study, the effect of gemcitabine in combination with pimasertib was assessed using human pancreatic cancer cell lines and an orthotopic mouse model of pancreatic tumor. The results obtained indicated that the addition of a MEK inhibitor to gemcitabine treatment enhanced its activity by inducing RRM1 degradation, and this can be taken into consideration for the design of future clinical trial.

Materials and Methods

Reagents

The MEK1/2 inhibitor pimasertib (AS703026 or MSC1936369B) was kindly provided by EMD Serono. The PI3K inhibitor GDC-0942 was purchased from Stratech Scientific Ltd. Both reagents were dissolved in DMSO to make a 10 mmol/L stock solution and were stored at ~20°C. Gemcitabine was a gift from the University College Hospital McMillan Cancer Center (London, UK). The following reagents were used: thiazoyl blue tetrazolium bromide (MTT), cycloheximide (Sigma-Aldrich), and MG132 (Calbiochem). For immunoblotting analysis, the following antibodies were used: anti-β-actin and anti-calnexin as loading controls; anti-RRM1, anti-p-ERK, anti-AKT, anti-ERK, anti-K48, anti-MEK1, anti-MEK2 (Cell Signaling Technology) and anti-MDM2 (Santa Cruz Biotechnology). For immunoprecipitation analysis, antibodies against RRM1 (Abcam) and MDM2 (Santa Cruz Biotechnology) were used. For immunohistochemistry (IHC), antibodies against RRM1 (Proteintech), CC3, P-ERK (Cell Signaling Technology), Ki67 and p-AKT (Abcam) were used. Smart-pool siRNA for human RRM1, MEK1, MEK2, MDM2, and nontargeting controls were obtained from Thermo Scientific.

Cell line and culture conditions

PANC-1, BxPC-3, MIAPaCa-2, and SUIT-2 human cell lines were purchased from the ATCC. TB32048 cell lines, derived from murine KPC tumors, were a kind gift of Dr. David Tuveson. PANC-
Immunoblotting and immunoprecipitation

Protein extracts were prepared with the Celllytic M cell lysis reagent (Sigma-Aldrich). Thirty-five micrograms of protein was denatured by heating for 5 minutes at 95°C in sample buffer containing 100 mmol/L Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue (Life Technologies) and resolved on a 4%–12% Bis-Tris NuPAGE gel (Life Technologies). Proteins were subsequently transferred to polyvinylidene difluoride membranes (Immobilon-P transfer membrane; Millipore) in 1× Tris-glycine 20% methanol transfer buffer. Membranes were blocked for 1 hour at room temperature in blocking buffer containing 5% BSA (Sigma-Aldrich) in 1× TBS, 0.1% Tween-20. All primary antibodies were incubated overnight at 4°C. Anti-rabbit or mouse IgG, HRP-linked Antibody (Cell Signaling Technologies) were used to detect primary antibody binding. The binding was visualized by enhanced chemiluminescence (Amersham) on autoradiography film (Kodak-X-Omat).

To investigate protein–protein interaction and RRM1 ubiquitination, cells were plated at 2×10⁶/mL (15-cm dishes) and allowed to proliferate overnight before treatment. Cells were then pretreated for 1 hour with the proteasome inhibitor MG132 followed by treatment with pimasertib. Four hours later, approximately 10⁵ cells per dish were lysed in 500 μL of Celllytic M Cell lysis reagent (Sigma) supplemented with protease and phosphatase inhibitors (Roche) and Benzonase (Merck) according to manufacturer's protocol. Seven hundred micrograms of protein sample was incubated with 1 μg of anti-RRM1 (abcam) or anti-MDM2 antibody (Santa Cruz Biotechnology) coupled to protein A beads and left rotating at 4°C overnight. Immunoprecipitations were eluted by boiling in SDS-PAGE loading buffer for 5 minutes at 100°C and analyzed by immunoblotting for RRM1, K-48, and MDM2.

Cell viability and apoptosis assay

Four thousand cells per well were seeded in a clear, flat-bottom 96-well plate (Corning). The following day, cells were treated with gemcitabine or pimasertib for 48 hours and pretreated with pimasertib for 4 hours followed by gemcitabine for 48 hours, before harvesting. All drugs were diluted in cell culture media. Following drug treatments, cells were incubated with 20 μL/well of MTT (5 mg/mL) for 4 hours at 37°C. Formazan crystals were solubilized in 200 μL DMSO, and the absorbance was measured at 540 nm with the Varioskan Plate reader.

Apoptosis was measured by assessing caspase-3/7 enzyme activity with the Caspase 3/7 Glo assay (Promega). Luminescence was measured with the Varioskan Flash Multimode Reader (Thermo Scientific), and values were normalized to untreated control and presented as fold increase of control.

Calcusyn software analysis

The fraction affected (Fa) obtained from the single drug treatments or from the combination assays were used for the calculation of the combination indices (CI) with the Calcusyn Software (Biosoft) according to the non-fixed ratio design. CI < 0.8 indicate synergism, CI between 0.8 and 1.2 indicate an additive effect, CI > 1.2 indicate antagonism (24).

Quantitative reverse transcription PCR

RNA extraction from cells was carried out by using RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from 1 μg RNA by random primed reverse transcription using Omniscript RT kit (Qiagen). RRM1 (101040698_m1) and RRM2 (00357247_g1), Assays-on-demand were obtained from Applied Biosystems. PCRs were done in a 20 μl reaction volume containing 10 μl of 2× buffer/enzyme mix, 1 μl of 20× assay mix, 1 μl of 20× GAPDH (Hs02758991_g1) endogenous control assay mix, and 1 μl input cDNA. Assays were run on an Applied Biosystems 7500 Sequence Detection System, and results were analyzed by the standard curve method. Data were normalized to Universal Human Reference RNA (Agilent 750500).

siRNA transfection

Cells were plated in a 6-well plate at a concentration of 1×10⁶/mL. Twenty-four hours after plating, cells were then transfected with 50 nmol/L of ON-TARGETplus SMARTpool siRNA targeting RRM1, MEK1, MEK2, or MDM2 and with non-targeting siRNA (scrambled) for 72 hours, according to the Dharmafekt transfection reagent protocol (Dharmacon).

Syngeneic orthotopic mouse model of pancreatic cancer

Four to 8-week-old female C57BL/6 mice were used in in vivo experiments according to the Animal Research Ethics and United Kingdom Coordination Committee on Cancer Research Guidelines and Home Office Regulations (Project license PPL70/7411). All animals were housed under specific pathogen-free conditions, and all procedures involving animals were conducted according to the requirements of the United Kingdom Home Office Animals (Scientific Procedures) Acts, 1986. After acclimatization for 1 week, 1×10⁵ TB32048 tumor cells, a pancreatic cancer cell line syngeneic for C57BL/6 mice that was derived from the KPC genetic pancreatic cancer model (LSLTrp53R172H/+, LSLKrasG12D/+, LSLTrp53R172H/+ Pdcd1-Cre), generously given by David Tuveson, in 30 μL of PBS: Matrigel (1:1) were orthotopically injected into the pancreas. After tumors were established for 2 weeks, gemcitabine was administered intraperitoneally at dose of 80 mg/kg, twice a week. Pimasertib was suspended in 0.5% carboxy methylcellulose and 0.25% Tween-20 and injected by oral gavages once daily at dosage of 5 mg/kg. Twelve days after treatment, mice were sacrificed. Tumors were excised, weighed, and fixed in paraformaldehyde for IHC analysis.

IHC

For IHC analysis, 4-mm paraffin sections underwent automated dewaxing (Leica Bond Dewax AR9222), and endogenous peroxidase was blocked using 3-4% (v/v) hydrogen peroxide (part of Leica Bond Refine Polymer Kit, D93800). Automated antigen retrieval was then performed on the sections. For Ki67, cleaved caspase-3, and RRM1, Leica Bond ER2 (EDITA-based, p19, AR9640) was applied to the slides and they were heated to 100°C (30 minutes for Ki67, p-AKT, and CC3 and 10 minutes for RRM1). No antigen retrieval was used for p-ERK. The antibody was used on the slides obtained from mice pancreatic tumors at a dilution of 1:100 (Ki67), 1:1,000 (cleaved caspase-3), 1:200 (RRM1 and p-
ERK), with 15 minutes incubation or 1:200 (p-AKT), with 30-minute incubation. Signal visualization was performed using Bond Polymer (Anti-rabbit Poly-HRP-IgG) for 8 minutes. 3,3’-Diaminobenzidine (DAB) was applied for 10 minutes, and then Bond DAB Enhancer (Copper Sulfate-based, AR9432) was applied for 5 minutes. Cell nuclei were counterstained with hematoxylin. The Leica Bond Polymer Detection Kit (DS9800) was used for peroxidase blocking, visualization, and counterstaining. Bond Wash (AR9590) was used for all washing steps between reagent steps.

IHC quantification

Immunostaining for RRM1, p-AKT, and p-ERK was assessed in at least five fields at 400× magnification. Immunoreactivity was evaluated semiquantitatively on the basis of staining intensity and proportion. The proportion of staining was scored from 0 to 3 as follows: 3, >50% of cells positive; 2, 10%–49%; 1, <10%. Intensity of staining was scored from 0 to 3 (0, absent; 1, weak; 2, moderate; 3, intense). The immunoreactive score for each sample was determined by multiplying the intensity and the proportion of stained cells. Analysis was undertaken blindly without knowledge of treatment variables.

Statistical analysis

The one-way ANOVA, two-way ANOVA with Bonferroni postests were used to calculate statistical significance for the in vitro experiments. The Student t test was used to calculate statistical significance of tumor weight. *P < 0.05, as calculated by GraphPad Prism (version 6.0; GraphPad Software Inc.), was considered statistically significant.

Results

The MEK1/2 inhibitor pimasertib enhances the antiproliferative and apoptotic effects of gemcitabine in PANC-1 and BxPC-3 cells

The sensitivity to gemcitabine and the MEK1/2 inhibitor pimasertib on a panel of pancreatic cancer cell lines was measured by the MTT assay (data not shown). Pimasertib acts by binding to an allosteric pocket of MEK thus preventing phosphorylation of downstream kinase ERK (25). PANC-1 and BxPC-3 cells were treated with pimasertib, and total protein lysates were immunoblotted to probe modulation of ERK activation after MEK inhibition. The addition of 0.5 μmol/L pimasertib effectively suppressed ERK phosphorylation over a 48-hour time course (Fig. 1A). The antiproliferative effect of gemcitabine in combina-

| Table 1. Combination indices (CI) of gemcitabine and pimasertib combination in PDAC cell lines |
|---------------------------------|-----------------|----------------|---|
| Pimasertib | Gemcitabine | Combination |
|            |            | index |
| **PANC-1** |            |    |
| 500 nmol/L | 5 nmol/L | 0.28 |
| 500 nmol/L | 25 nmol/L | 0.53 |
| 500 nmol/L | 50 nmol/L | 0.59 |
| **BxPC-3** |            |    |
| 500 nmol/L | 5 nmol/L | 0.16 |
| 500 nmol/L | 25 nmol/L | 0.36 |
| 500 nmol/L | 50 nmol/L | 0.51 |
| **Pimasertib + gemcitabine** |            |    |
| **PANC-1** |            |    |
| 500 nmol/L | 5 nmol/L | 2.7 |
| 500 nmol/L | 25 nmol/L | 1.4 |
| 500 nmol/L | 50 nmol/L | 1.6 |
| **BxPC-3** |            |    |
| 500 nmol/L | 5 nmol/L | 6.95 |
| 500 nmol/L | 25 nmol/L | 3.60 |
| 500 nmol/L | 50 nmol/L | 2.52 |

Figure 1.

The MEK1/2 inhibitor pimasertib sensitizes PDAC cell lines to gemcitabine-induced cytotoxicity. A, effect of pimasertib treatment on ERK signaling. PANC-1 and BxPC-3 cells were treated with increasing concentrations (0.1, 0.5, 1 μmol/L) of pimasertib for 24 hours or with 1 μmol/L pimasertib and extracted at different time points (30 minutes to 48 hours). Total lysates were analyzed by immunoblotting with the indicated antibodies, and calnexin was used as a loading control (Table 1). The MTT assay was used to assess the effect of a 4-hour pretreatment with 500 nmol/L pimasertib followed by a 48-hour exposure with increasing concentrations of gemcitabine (5, 25, 50 nmol/L) or simultaneous treatment of 500 nmol/L pimasertib with increasing concentrations of gemcitabine (5, 25, 50 nmol/L) for 48 hours, on the growth of PANC-1 and BxPC-3 cells. CIs describing drug combinations were calculated from the mean Fa of three independent experiments. B, effect of gemcitabine, pimasertib, and their combination on apoptosis. Caspase-3/7 activity was used to detect apoptosis in PANC-1 and BxPC-3 cells after 48-hour treatment with 500 nmol/L pimasertib, 50 nmol/L gemcitabine and 4-hour pretreatment with 500 nmol/L pimasertib followed by 48-hour treatment with 50 nmol/L gemcitabine. Results are presented as fold increase to untreated sample and are shown as mean ± SD (n = 3). There was a significant increase in cell death when gemcitabine was combined with pimasertib in PANC-1 (**, P < 0.001) and BxPC-3 (***, P < 0.001) cells.
Figure 2.
MEK inhibition reduces RRM1 protein levels through a posttranslational modification. A, PANC-1, BxPC-3, SUIT-2, and MIAPaCa-2 cells were treated for 24 hours with 1 μmol/L pimasertib, 50 nmol/L gemcitabine alone, or in combination with 1 μmol/L pimasertib. Immunoblotting analysis was performed on whole-cell lysates. Calnexin was used as a loading control. Results are representative of three independent experiments. B, PANC-1 and BxPC-3 cells were transfected with RNAi targeting MEK1 and MEK2 for 72 hours. Whole-cell lysates were analyzed by immunoblotting with the indicated antibodies, and calnexin was used as a loading control. C, RNA extraction was performed in BxPC-3 cells after 24-hour treatment with pimasertib. mRNA levels of RRM1 and RRM2 were measured by real-time PCR and normalized to the GAPDH control. Each experiment was repeated in triplicate, and results are presented as mean ± SD (**, P < 0.01).

(Continued on the following page.)
nmol/L pimasertib for 4 hours followed by 50 nmol/L gemcitabine for 48 hours enhanced gemcitabine-induced apoptosis by increasing the levels of caspase-3/7 enzyme activity from 3.78 ± 0.11- to 5.64 ± 0.06-fold in the BxPC-3 and from 1.55 ± 0.02- to 2.62 ± 0.1-fold in the PANC-1 cells (Fig. 1B). In contrast, simultaneous combination of the two drugs did not show a significant reduction of apoptosis in BxPC-3 cells (from 2.96 ± 0.78- to 3.4 ± 0.48-fold; Supplementary Fig. S1B). The effect of pimasertib on G1 arrest explains antagonism observed for the simultaneous schedule, as gemcitabine acts predominantly on cells in S-phase (data not shown).

To confirm that pimasertib sensitized gemcitabine due to on-target inhibition of MEK protein, knockdown of MEK1 and MEK2 using gene-specific siRNAs was performed. BxPC-3 cells were transfected with 50 nmol/L of specific MEK1 and MEK2 siRNA or nontargeting siRNA (scrambled) for 72 hours followed by a 48-hour treatment with 100 μg/mL cycloheximide. The addition of gemcitabine to MEK1 or MEK2 knockdown cells enhanced its efficacy by reducing proliferation of BxPC-3 cells from 69.03 ± 0.77 to 24.46 ± 1.37 (mean ± SD; MEK1 siRNA) and to 26.04 ± 2.98 (mean ± SD; MEK2 siRNA; Fig. 1C).

Pimasertib reduces RRM1 protein levels

To investigate how pimasertib modulates gemcitabine efficacy, the effects on genes involved in gemcitabine response were analyzed. Among the genes implicated in gemcitabine resistance and sensitivity (data not shown), a significant reduction in RRM1 protein was observed in BxPC-3, PANC-1, SUIT-2, and MIAPaCa-2 cells after 24-hour treatment with pimasertib (Fig. 2A). RRM1 is a target of gemcitabine, whose low expression is associated with improved response after gemcitabine treatment in patients with NSCLC and PDAC (27, 28). Reduced RRM1 protein expression was seen after MEK1 and MEK2 siRNA knockdown in BxPC-3 and PANC-1 cells (Fig. 2B). The different MEK1/2 inhibitor (AS703988) also induced downregulation of RRM1 protein after 24-hour treatment (Supplementary Fig. S2A), but the same effect did not occur when cells were exposed to an EGFR inhibitor (gefitinib; Supplementary Fig. S2B). These results indicate that the effect on RRM1 protein expression is specifically dependent on MEK inhibition.

Expression of RRM1 protein is constant during the cell cycle, whereas RRM2 protein expression fluctuates, reaching a peak in S-phase (29). To assess whether RRM1 downregulation is linked to a cell-cycle effect after pimasertib treatment, mRNA levels of RRM1 and RRM2 were analyzed by PCR. While the mRNA levels of RRM2 decreased after 24-hour treatment with pimasertib, mRNA RRM1 levels were not significantly altered in BxPC-3 cells (Fig. 2C). In contrast, immunoblotting analysis revealed a significant downregulation of RRM1 protein levels following only 4-hour treatment with pimasertib in BxPC-3 and PANC-1 cells, an effect that was not observed on RRM2 protein expression (Fig. 2D). Reduction of RRM1 protein after short treatment with pimasertib was also found in MIAPaCa-2 and SUIT-2 cells (Supplementary Fig. S2C). Importantly, pretreatment with the proteasome inhibitor MG132, which blocks the catalytic activity of the proteasome, impaired the ability of pimasertib to reduce RRM1 expression in BxPC-3 cells, suggesting that RRM1 is subjected to proteasomal degradation. Finally, BxPC-3 cells were incubated for 1 hour with...
Pimarsertib induces MDM2-mediated polyubiquitination and degradation of RRM1

Our data suggest that pimarsertib sensitizes pancreatic cancer cells to gemcitabine by reducing protein expression of RRM1. However, there may be other mechanisms by which pimarsertib affects gemcitabine efficacy. Ubiquitin chains are formed through various lysine residues. In particular, ubiquitin proteins attached to each other through lysine residue at position 48, target proteins for proteasomal degradation (30). To understand the mechanism of the decrease in RRM1 expression induced by MEK inhibition, cells were subjected to immunoprecipitation. RRM1 was immunoprecipitated from cells lysates derived from cells that had been pretreated for 1 hour with MG132, followed by 4-hour treatment with pimarsertib and subjected to immunoblotting with an antibody against Lys-48. Pretreatment with MG132 produced a ladder of bands at high molecular weights whose intensity increased after pimarsertib treatment in BxPC-3 and PANC-1 cells, suggesting that pimarsertib enhances RRM1 polyubiquitination through Lys-48–mediated linkage (Fig. 3A).

P53R2 is a p53-regulated subunit of the RR complex, which is upregulated upon DNA damage. Recent data have shown that the P53R2 subunit is polyubiquitinated and degraded by the E3 ligase MDM2 that is responsible for the proteasomal degradation of P53 (31). Depletion of MDM2 by siRNA impaired RRM1 downregulation induced by pimarsertib in BxPC-3 cells, compared with cells transfected with scrambled (Fig. 3B). Next, to determine whether RRM1 can interact with MDM2, co-immunoprecipitation was performed. Endogenous RRM1 bound to MDM2, and there was increased RRM1 binding after treatment with pimarsertib (Fig. 3C). Nutlin-3 is an MDM2 antagonist that inhibits the association between p53 and MDM2 in cells with wild-type p53, thus resulting in p53 accumulation. Recent studies have demonstrated that nutlin-3 is active in mutant p53 cells. For example, recent findings have demonstrated that nutlin-3 disrupted the binding of E2F1 with MDM2, thereby increasing E2F1 transcriptional activity (32). We were interested at investigating whether nutlin-3 could alter the effect induced by pimarsertib on RRM1 stability by inhibiting its binding with MDM2. Addition of 1 μmol/L nutlin-3 to pimarsertib on BxPC-3 cells (mutant p53 cells) inhibited RRM1 downregulation induced by pimarsertib (Fig. 3D), indicating that nutlin-3 blocks MDM2 association with RRM1. Treatment with p53 siRNA did not affect the downregulation of RRM1 induced by pimarsertib, suggesting that this effect is not dependent on p53 (Supplementary Fig. S4A).

MEK inhibition reduces RRM1 protein levels through a feedback loop activation of AKT

MEK inhibitors can induce upregulation of AKT phosphorylation through feedback regulation (33). In addition, AKT has been reported to phosphorylate MDM2 at Ser166 and Ser186 and enhance MDM2-mediated ubiquitination of p53 (34). To elucidate the mechanism of MDM2 actvation by pimarsertib, BxPC-3 and PANC-1 cells were treated for 4 hours with pimarsertib, GDC-0942, a PI3K inhibitor, and a combination of pimarsertib with GDC-0942 (a PI3K inhibitor). The addition of the PI3K inhibitor impaired RRM1 degradation induced by pimarsertib (Fig. 4A). The same effect was observed in SUIT-2 cells (Supplementary Fig. S4B). It is important to note that pimarsertib increased AKT phosphorylation as shown by the p-AKT/t-AKT ratio (Fig. 4B). These results show that MEK inhibition may lead to feedback activation of AKT, which induces proteasomal degradation of RRM1.

siRNA knockdown of RRM1 enhances gemcitabine efficacy

Several studies have demonstrated that RRM1 overexpression is associated with gemcitabine resistance in NSCLC and PDAC (15, 16). To confirm results of previous studies, BxPC-3 cells were treated with specific siRNA for RRM1 or scrambled (Fig. 5A). After 72 hours, cells were harvested and treated for 48 hours with gemcitabine. The effect on cell viability and apoptosis upon gemcitabine treatment in RRM1-transfected cells was evaluated with MTT and cleaved caspase 3 Glo assays. RRM1 knockdown cells showed a statistically significant increase in gemcitabine cytotoxicity by reducing viability from 69.09 ± 2.34 to 41.16 ± 10.45 (mean ± SD) in BxPC-3 cells (Fig. 5B) and enhancing apoptosis from 6.1 ± 0.8 to 9.4 ± 1.3 in BxPC-3 cells (Fig. 5C).

Combined treatment of pimarsertib with gemcitabine induced tumor growth delay in an orthotopic model of pancreatic cancer

The effect of pimarsertib on RRM1 protein expression and AKT activation was evaluated in the TB32048 cell line. Immunoblotting analysis showed inhibition of ERK phosphorylation and reduced RRM1 expression, with an increase of AKT phosphorylation after 4- and 24-hour treatment with pimarsertib (Fig. 6A). The results obtained were consistent with what was observed in the human pancreatic cancer cell lines.

To determine whether pimarsertib enhances gemcitabine efficacy in vivo, TB32048 mouse cells derived from the pancreatic mouse model KPC (LSL-KRASG12D/−/LSLTrp53R172H/+Pdx1-Cre) were orthotopically implanted in 6-week-old C57/B6 black female mice. A 5-day treatment cycle was performed. Mice were treated with vehicle, single-agent gemcitabine intraperitoneally 80 mg/kg (twice a week), single-agent pimarsertib via oral gavage at 5 mg/kg (daily), or with pimarsertib 5 mg/kg (daily) followed 4 hours later by gemcitabine 80 mg/kg (twice a week). After two treatment cycles, mice were sacrificed, tumor excised, weighed, and sectioned for histopathologic analysis. Mice treated with single-agent pimarsertib or gemcitabine had no significant difference in tumor weight compared with the vehicle cohort. In contrast, combination treatment showed tumor sizes significantly lower than treatment with either gemcitabine (∗, P < 0.05) or pimarsertib alone (∗∗, P < 0.01). Representative gross images of tumor are shown (Fig. 6B). The effect observed was due to lack of growth, as expected, as the treatment period in these experiments was brief to examine dynamic effects on gene expression as in in vitro studies. All drug treatments regimens were well-tolerated in mice. No signs of acute toxicities were observed.

The average tumor volumes of the combination group were not significantly different before and after treatment (P > 0.05; Fig. 6C). The addition of 15 mg/kg pimarsertib to 80 mg/kg gemcitabine also significantly reduced tumor weight compared with gemcitabine alone (∗∗, P < 0.01) and pimarsertib alone (∗, P < 0.05) in TB32048 xenograft mice (Supplementary Fig. S5A). Statistical analysis was performed using the Student t test.

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Figure 3. Pimasertib induces MDM2-mediated ubiquitination and degradation of RRM1. 

A, PANC-1 and BxPC-3 cells were pretreated for 1 hour with 1 μmol/L MG132 followed by 1 μmol/L pimasertib for 4 hours. Immunoprecipitation was performed using anti-RRM1 antibody or anti-IgG antibody and immunoblotted with anti-RRM1 and anti-K48 antibodies. Input lysates were blotted with RRM1 antibody. Results are representative of three independent experiments. 

B, BxPC-3 cells were treated with specific RNAi targeting MDM2 (50 nmol/L) or scrambled for 72 hours, followed by 4-hour treatment with 1 μmol/L pimasertib. Immunoblotting analysis was performed on whole-cell lysates with the indicated antibodies. 

C, BxPC-3 cells were pretreated for 1 hour with 1 μmol/L MG132 followed or not by 4-hour treatment with 1 μmol/L pimasertib, and cell lysates were immunoprecipitated (IP) with control IgG, anti-RRM1, or anti-MDM2 antibody. IP complexes were immunoblotted with the indicated antibodies. 

D, BxPC-3 cells were cotreated with 1 μmol/L nutlin-3 and 1 μmol/L pimasertib for 24 hours, and protein lysates were analyzed by immunoblotting with the indicated antibodies. Calnexin was used as a loading control. Results are representative of two independent experiments.
IHC analysis was done to determine the effect of pimasertib and gemcitabine on TB32048 xenograft tumors. Hematoxylin and eosin (H&E) staining was performed to determine tissue morphology. Proliferation and apoptosis were analyzed by staining for Ki67 and cleaved caspase-3. An increase of the percentage of cleaved caspase-3–positive cells and a decrease of Ki67 staining was observed in mice treated with pimasertib/gemcitabine regimen compared with vehicle. Specific target inhibition was seen in tumors harvested from pimasertib-treated mice, as evidenced by a significant decrease of p-ERK staining. Importantly, the expression of RRM1 was reduced in pimasertib-treated mice, a result that was comparable to the results obtained in our in vitro studies. Finally, an increase in AKT phosphorylation was found upon pimasertib treatment in TB32048 xenograft tumors. Representative images of the staining are shown in Fig. 6D. Quantification of RRM1, p-ERK, and p-AKT staining in tumor tissues treated was performed (Fig. 6E).

Discussion

PDAC is a malignancy that is resistant to most conventional chemotherapeutic agents. Gemcitabine represents one of the standard treatments for advanced PDAC (35), exerting its cytotoxic effect by inhibition of DNA duplication, leading to cell death (36). In addition, gemcitabine inactivates RRM1 through covalent binding, thus reducing the amount of dNTP pools available for new DNA synthesis and repair (9). The documented, although limited, activity of the nucleoside analogue gemcitabine has made this agent a major component of regimens used in the advanced setting. Both primary and acquired resistance are common features in patients with PDAC treated with gemcitabine. The alteration of chemosensitivity by agents that modulate the gemcitabine metabolic pathway has been shown in several studies. Recent research on a pancreatic cancer mouse model found that the combination of nab-paclitaxel with gemcitabine induced a greater effect on tumor regression compared with gemcitabine alone through reactive oxygen species (ROS)-mediated reduction of CDA protein expression (37).

Other biomarkers that could impact gemcitabine efficacy have been investigated. For example, a preclinical study demonstrated that the expression of dCK is correlated with gemcitabine response following radiation in bladder cancer (38). Phase II studies in advanced NSCLC have shown that RRM1 expression is inversely correlated with patient’s response to gemcitabine-based therapies (27, 39). Furthermore, high expression of RRM1 is associated with worse survival after gemcitabine treatment in patients with pancreatic cancer, suggesting a major role for RRM1 in intrinsic resistance to gemcitabine (40). Several
gemcitabine-based combinations have been evaluated in clinical trials and resulted in improved antitumor activity compared with monotherapy treatment (4, 5).

Genetic alterations of the RAS/RAF/MEK/ERK signaling pathway are frequently found in PDAC, particularly, mutation of the KRAS gene, which contributes to tumor growth and chemoresistance (41); inhibition of MEK1/2 has become an attractive target in human cancers characterized by aberrantly activated MAPK signaling pathway. One of the benefits of using MEK inhibitors is represented by the fact that most of them are not competitive with ATP but bind to an adjacent allosteric site that locks MEK into a catalytically inactive conformation, thereby preventing any side effects associated with inhibition of other protein kinases (18).

Inhibitors of MEK protein have been developed to target this pathway but have shown limited efficacy when used in monotherapy in many tumors where they have been tested (42). Pimasertib (AS703026 or MSC1936309B) is a potent allosteric MEK1 and MEK2 inhibitor; in vitro and in vivo effects of pimasertib alone or in combination have been evaluated in several human cancers where MAPK pathway is commonly deregulated and have shown potent antitumor activity (22, 23). In this study, we found that one mechanism by which pimasertib modulates gemcitabine activity is through a reduction in RRM1 protein expression, an important biomarker for gemcitabine resistance both in PDAC and NSCLC (16, 27). In particular, addition of pimasertib to gemcitabine significantly increased its antiproliferative and apoptotic effects in pancreatic cancer cell lines, an effect that was dependent on the schedule of administration. In a study of the MEK inhibitor AZD6244 using biliary cancer models, the importance of scheduling was demonstrated with simultaneous combination of AZD6244 and gemcitabine leading to G1 accumulation, antagonizing gemcitabine activity, which is S-phase dependent (43). Ribonucleotide reductase is composed of two subunits, RRM1 and RRM2, the latter being cell cycle regulated and exhibiting its highest expression during S-phase (12). Our data showed that RRM1 mRNA expression levels were not reduced by addition of pimasertib. In contrast, a striking reduction of RRM1 protein was observed after pimasertib treatment within 4 hours, an effect that was impaired by the addition of the proteasome inhibitor MG132. The protein synthesis inhibitor cycloheximide reduced protein levels of RRM1, confirming that RRM1 downregulation occurs through posttranslational modification. Downregulation of RRM1 protein did not occur in response to an EGFR inhibitor (gefitinib) but was observed in MEK1/2 knocked down cells, indicating that this effect is specifically dependent on MEK inhibition. A recent study has shown that RRM1 is polyubiquitinated and identified RNF2 and Bmi1 as ubiquitin ligases to be involved in RRM1 degradation by the proteasome (44). Our study showed enhancement of RRM1 polyubiquitination through Lys-48-mediated linkage upon pimasertib treatment.

MDM2 is a E3 ubiquitin ligase that targets p53 for proteasomal degradation (45). A recent study found that MDM2 is involved in the regulation of RR subunit P53R2 activity (31). Here we demonstrated that MDM2 directly interacts with RRM1 and that depletion of MDM2 by specific siRNA impaired RRM1 downregulation induced by pimasertib. Nutlin-3 is an MDM2 antagonist that binds p53, thus disrupting the interaction between p53 and MDM2, which results in accumulation of p53 (46). However, recent studies have demonstrated a p53-independent role of nutlin-3 (32). Our results showed that the addition of nutlin-3 to pimasertib inhibited RRM1
downregulation, confirming the involvement of MDM2 in the degradation of RRM1.

The RAF/MEK/ERK and PI3K/AKT signaling pathways can interact with each other (33). In this study, we found that pimasertib induced p-AKT activation and that the addition of the PI3K inhibitor GDC-0941 to pimasertib treatment impaired reduction of RRM1, implying a role of the PI3K/AKT pathway in the RRM1 degradation induced by MEK inhibition. Other studies have shown that AKT interacts with and activates MDM2 leading to its nuclear translocation and degradation of p53 (47). On the basis of our findings, we hypothesize that the AKT activation induced by MEK inhibition may trigger MDM2-mediated degradation of RRM1. Further experiments will need to be performed to validate this hypothesis.

Figure 6.
The combination of pimasertib with gemcitabine induces tumor growth delay. A, TB32048 mouse cells were treated for 4 and 24 hours with 1μmol/L pimasertib. Total cell lysates were immunoblotted using the indicated antibodies, and calnexin was used as a loading control. The average ratio of p-AKT over t-AKT from three independent experiments was calculated and plotted (bar graph).

B, TB32048 tumor-bearing mice were treated for 12 days with vehicle, 5 mg/kg pimasertib (daily) by oral gavage, gemcitabine 80 mg/kg (twice a week) intraperitoneally, or a sequential combination of pimasertib 5 mg/kg followed by gemcitabine 80 mg/kg. Gross images from representative excised tumors are shown at the bottom. After sacrifice, tumor weight was measured. The Student t test was used to determine the statistical significance of differences in tumor weight (*, *P < 0.01; **, *P < 0.001).

C, tumor volumes were measured by ultrasound analysis before and after treatment. One-way ANOVA was performed for statistical analysis (***, *P < 0.001). (Continued on the following page.)
In accordance with previous published data, we confirmed that a correlation exists between RRM1 levels and resistance to gemcitabine (40, 44). Gemcitabine-induced apoptosis was increased and proliferation reduced upon depletion of RRM1 with specific siRNA, confirming its key role in gemcitabine resistance.

Figure 6. (Continued.) D, IHC staining from tumors treated with the indicated drugs and vehicle. Paraformaldehyde-fixed paraffin sections were incubated with anti-Ki67, anti-cleaved caspase-3, anti-p-ERK, anti-p-AKT, and anti-RRM1 antibody. H&E staining was performed. Representative images are illustrated. E, quantification of IHC staining for p-ERK, RRM1, and p-AKT. Data, mean ± SD. One-way ANOVA was performed for statistical analysis (**, P < 0.01; ***, P < 0.001).
Finally, our study provided in vivo evidence that gemcitabine, when combined sequentially with pimasertib, induced significant tumor growth delay, along with reduction of RRM1 expression, in an orthotopic model of pancreatic cancer. Together, these data strongly suggest that combining MEK inhibitors with gemcitabine is a potential strategy to improve its efficacy and that RRM1 expression plays a crucial role as a marker of resistance to gemcitabine therapy in human pancreatic cancer.

These preclinical investigations were performed after the phase I/II trial examining pimasertib in combination with gemcitabine in patients with PDAC was initiated to elucidate further the underlying mechanism by which pimasertib sensitizes pancreatic cancer cells to gemcitabine. During the course of this preclinical study, it was demonstrated that the schedule of administration significantly affected the mechanism of the combination and, consequently, the efficacy of the combination in vitro. While clinical data for combinations of MEK inhibitors, like pimasertib, with gemcitabine have been disappointing up to now (48), we suggest that a modified approach may be considered in future studies. The importance of scheduling as a factor by which MEK inhibition increases gemcitabine sensitivity will inform future clinical investigations for combinations and schedules of gemcitabine and MEK inhibitors in the treatment of PDAC.

Disclosure of Potential Conflicts of Interest
F. Vena reports receiving other commercial research support from Merck Serono. D. Hochhauser reports receiving a commercial research grant from Merck Serono. No potential conflicts of interest were disclosed by the other authors.

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The MEK1/2 Inhibitor Pimasertib Enhances Gemcitabine Efficacy in Pancreatic Cancer Models by Altering Ribonucleotide Reductase Subunit-1 (RRM1)

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