THE MEK1/2 INHIBITOR PIMASERTIB ENHANCES GEMCITABINE EFFICACY IN PANCREATIC CANCER MODELS BY ALTERING PROTEIN LEVELS OF RIBONUCLEOTIDE REDUCTASE SUBUNIT-1 (RRM1)

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Disclosure of Potential Conflicts of Interest

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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 signalling pathway by direct inhibition of MEK1/2 proteins is a promising therapeutic strategy, since 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 was 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. Pre-treatment with MG132 impaired reduction of RRM1 protein induced by pimasertib, suggesting RRM1 is degraded post-translationally. Immunoprecipitation indicated enhanced MDM2-mediated polyubiquitination of RRM1 through lys48-mediated linkage following pimasertib treatment, an effect in part mediated by AKT. Finally, the combination treatment with pimasertib and gemcitabine caused significant tumor growth delays in an orthotopic pancreatic cancer model, with RRM1 down-regulation in pimasertib-treated mice.
**Conclusion:** These results confirm an important role of RRM1 in gemcitabine response and indicate MEK as a potential target to sensitize gemcitabine therapy for PDAC.

**Statement of translational relevance**

Gemcitabine represents an important treatment for locally advanced and metastatic pancreatic ductal adenocarcinoma (PDAC), although effects on survival are modest. The optimisation of chemotherapy by combination with novel agents is therefore of great importance and the mechanisms of these interactions need investigation. In this preclinical study, the combination of gemcitabine with the MEK1/2 inhibitor pimasertib was investigated in pancreatic cancer models. Enhancement of gemcitabine activity was observed with pimasertib treatment. A significantly reduced level of Ribonucleotide Reductase Large Subunit-1 (RRM1), an important marker of gemcitabine sensitivity, was found following exposure of PDAC models to pimasertib. These results confirm an important interaction between pimasertib and gemcitabine and identify MEK as a potential target to sensitize gemcitabine therapy. Understanding the effects of MEK inhibition on gemcitabine sensitivity will inform clinical strategies for combining these agents in the treatment of PDAC.
INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease associated with poor prognosis, with few patients surviving one year after diagnosis (1). Gemcitabine, an anti-metabolite with similar structure to the nucleoside cytidine, (Gem), (2’, 2’-difluorodeoxycytidine: 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 six months (2).

Drug resistance is a common feature in PDAC patients treated with gemcitabine (3), increasing the need to develop combination therapies, which could enhance gemcitabine effectiveness. Among all gemcitabine-based combinations treatments tested in clinical trials, gemcitabine with abraxane, the albumin-bound formulation of paclitaxel, has shown improved overall and progression-free survival in metastatic PDAC (4). Additionally, in a randomized phase III trial, the chemotherapy regimen FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) significantly prolonged median overall survival compared to gemcitabine monotherapy in patients with metastatic pancreatic cancer (5).

Gemcitabine is a pro-drug, which is actively transported inside the cells through human equilibrative nucleotide transporters (hENTs) and transformed into its active form, 2’, 2’-difluorodeoxycytidine 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) (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) (7) (8). RNR is the rate-limiting step of DNA duplication (9), since 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 P53R2, a subunit dependent on P53 activity (9). The two subunits associate, forming an active holoenzyme (11). Levels of R1 protein are constant during the cell cycle; R2 levels oscillate during the cell cycle with a peak in S-phase (12).

From *in vitro* and clinical studies conflicting results have emerged regarding the biological 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, *in vitro* and clinical studies have demonstrated that RRM1 overexpression is associated with gemcitabine resistance in non-small cell lung cancer (NSCLC) and PDAC patients, with high levels of RRM1 expression being associated with 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 MAPKK proteins, dual-specificity kinases (17). There are seven MEK proteins identified involved in four different MAPK signalling pathways; only MEK1 and MEK2 belong to the RAS-RAF-ERK signalling 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 signalling pathway is frequently deregulated in human cancers. In particular, mutations of the K-RAS gene occur in almost 90% of PDAC and have been found in early precursor lesions of PDAC, denominated pancreatic intraepithelia 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. Additionally, 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 (MM) cell lines, MM patient cells and MM xenograft models and enhanced the cytotoxic effect of conventional anti-MM 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 MEK1/2 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 (Billerica, MA). The PI3K inhibitor GDC-0942 was purchased from Stratech Scientific Ltd. Both reagents were dissolved in DMSO to make a 10mM stock solution and were stored at -20°. Gemcitabine was a gift from the University College Hospital McMillan Cancer Center (London, UK). The following reagents were used: Thiazolyl 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-AKT; anti-AKT; anti-p-ERK; anti-ERK; anti-K48; anti-MEK1; anti-MEK2 (Cell Signaling Technology) and anti-MDM2 (Santa Cruz Biotechnology). For Immunoprecipitation analysis antibody against RRM1 (Abcam) and MDM2 (Santa Cruz Biotechnology) were used. For Immunohistochemistry, 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 non-targeting 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 American Type Culture Collection (ATCC; Manassas, VA, USA). TB32048 cell lines, derived from murine KPC tumors, were a kind gift of Dr. David Tuveson. PANC-1 and TB32048 cell lines were grown in Dulbecco's minimal essential medium (Autogen Bioclear), BxPC-3, MIAPaCa2 and SUIT-2 cell lines were grown in RPMI-1640 medium (Autogen Bioclear). All cells were
supplemented with 10% fetal bovine serum, 5% glutamine and 5% Penicillin/Streptomycin and incubated at 37°C in 5% CO₂.

Immunoblotting and Immunoprecipitation

Protein extracts were prepared with the CelLytic™ M cell lysis reagent (Sigma-Aldrich). 35 μg of protein were denatured by heating for 5 min at 95°C in sample buffer containing 100 mM Tris-Cl (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 1X-Tris-Glycine-20% Methanol transfer buffer. Membranes were blocked for 1h at room temperature in blocking buffer containing 5% BSA (Sigma-Aldrich) in 1x 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 × 10⁵/mL (15 cm dishes) and allowed to proliferate overnight before treatment. Cells were then pre-treated for 1 hour with the proteasome inhibitor MG132 followed by treatment with pimasertib. 4 hours later, approximately 10⁷ cells/dish were lysed in 500 μL of CelLytic M Cell lysis reagent (Sigma) supplemented with protease and phosphatase inhibitor (Roche) and Benzonase (Merck) according to manufacturer's protocol. 700 μg 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. IPs were eluted by boiling in SDS-PAGE loading buffer for 5 min at 100° and analysed by immunoblotting for RRM1, K-48, P-MDM2 and MDM2.

**Cell viability and apoptosis assay**

4,000 cells/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 pre-treated 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 20μL/well of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) (5mg/mL) for 4h at 37°. Formazan crystals were solubilised in 200μL of DMSO and the absorbance was measured at 540nm 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 1μg RNA by random primed reverse transcription using Omniscript RT kit (Qiagen). RRM1 (01040698_m1) and RRM2 (00357247_g1), Assays-on-demand were obtained from Applied Biosystems. PCRs were done in a 20 μL reaction volume containing 10μL of 2× buffer/enzyme mix, 1μL of 20× assay mix, 1 μLof 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 analysed by the standard curve method. Data were normalised to Universal Human Reference RNA (Agilent 750500).

siRNA transfection

Cells were plated in a 6-well plate at a concentration of 1 × 10^5/mL. Twenty-four hours after plating, cells were then transfected with 50nM of ON-TARGETplus SMARTpool siRNA targeting RRM1, MEK1, MEK2 or MDM2 and with non-targeting siRNA (scrambled) for 72h, according to the Dharmafect transfection reagent protocol (Dharmacon).

Syngeneic orthotopic mouse model of pancreatic cancer

Six- to eight-week-old female C57BL/6 mice were used in all 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, 1×10^5 TB32048 tumor
cells, a pancreatic cancer cell line syngeneic for C57BL/6 mice that was derived from the KPC genetic pancreatic cancer model (LSL-KRAS\(^{G12D/+}\) LSLTrp53\(^{R172H/+}\)Pdx1-Cre), generously given by Prof. David Tuveson,) in 30 μL of PBS: Matrigel (1:1) were orthotopically injected into the pancreas. After tumors were established for two weeks, gemcitabine was administered intraperitoneally at dose of 80 mg/kg, twice a week. Pimasertib was suspended in 0.5% carboxyl 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.

**Immunohistochemistry**

For immunohistochemical analysis, 4mm 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, DS9800). Automated antigen retrieval was then performed on the sections. For Ki67, Cleaved Caspase-3 and RRM1, Leica Bond ER2 (EDTA-based, pH9, AR9640) was applied to the slides and they were heated to 100 degrees Celsius (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), 1/1000 (Cleaved Caspase-3), 1/200 (RRM1and P-ERK), with 15 minutes incubation or 1/200 (p-AKT), with 30 minutes incubation. Signal visualization was performed using Bond Polymer (Anti-rabbit Poly-HRP-IgG) for 8 minutes. 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 haematoxylin. The Leica Bond Polymer Detection Kit (DS9800) was used for peroxidase
blocking, visualisation 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 semi-quantitatively based on 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 post-tests were used to calculate statistical significance for the in vitro experiments. Student’s 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.), were considered statistically significant.
Results

The MEK1/2 inhibitor pimasertib enhances the antiproliferative and apoptotic effect 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 MTT assay (data not shown). Pimasertib acts by binding 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 0.5µM pimasertib effectively suppressed ERK phosphorylation over a 48-hour time course (Fig 1A). The antiproliferative effect of gemcitabine in combination with pimasertib on PANC-1 and BxPC-3 cells was investigated. Sequential drug treatment consisting of a 4-hour pre-exposure to 500nM pimasertib followed by a 48-hour treatment with increasing concentrations of gemcitabine (5nM, 25nM, 50nM) enhanced the anti-proliferative effect of gemcitabine on PANC-1 and BxPC-3 cells. Combination indices (CI), which describe the interaction between drugs, were calculated using the methodology of Chou and Talalay, (26) with the CalcuSyn Software. At the three concentrations of gemcitabine tested, CI values describing the interaction with pimasertib are <0.8 and indicate synergism (CI=0.28 at 5nM CI=0.53 at 25nM, CI= 0.59 at 50nM gemcitabine in the PANC-1; CI=0.16 at 5nM, CI=0.36 at 25nM, CI= 0.51 at 50nM gemcitabine in BxPC-3) when pimasertib was administered 4h before gemcitabine. Simultaneous administration of gemcitabine with pimasertib resulted in CI values > 1.2 indicating a negative interaction (CI=2.7 at 5nM CI=1.4 at 25nM, CI= 1.6 at 50nM gemcitabine in the PANC-1; CI=6.95 at 5nM, CI=3.60 at 25nM, CI= 2.52 at 50nM gemcitabine in BxPC-3) (Table 1). Furthermore, a short treatment of gemcitabine (24 hours)
following 4h pre-treatment with pimasertib also showed a synergistic growth-inhibitory effect in BxCPC-3 cells (Fig S1A).

To examine the effect of drug combination on apoptosis, the Caspase 3-7 Glo assay was performed. Pre-treatment with 500nM pimasertib for 4 hours followed by 50nM gemcitabine for 48 hours enhanced gemcitabine-induced apoptosis by increasing the levels of caspase 3/7-enzyme activity from $3.78 \pm 0.11$ to $5.64 \pm 0.06$ fold in the BxPC-3 and from $1.55 \pm 0.02$ to $2.62 \pm 0.1$ 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 $2.96 \pm 0.78$ to $3.4 \pm 0.48$ fold) (Fig S1B). The effect of pimasertib on G1 arrest explains antagonism observed for the simultaneous schedule since 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 siRNA’s was performed. BxPC-3 cells were transfected with 50nM of specific MEK1 and MEK2 siRNA or non-targeting siRNA (scrambled) for 72 hours followed by a 48 hour treatment with 100nM gemcitabine. The addition of gemcitabine to MEK1 or MEK2 knock down cells enhanced its efficacy by reducing proliferation of BxPC-3 cells from $69.03 \pm 0.77$ to $24.46 \pm 1.37$ (mean ± SD)(MEK1 siRNA) and to $26.04 \pm 2.98$ (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 analysed. Among the genes analysed to be known to be involved 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 NSLC and PDAC patients (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 down-regulation of RRM1 protein after 24 hours treatment (Fig S2A) but the same effect did not occur when cells were exposed to an EGFR inhibitor (gefitinib) (Fig S2B). These results indicated 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 if RRM1 down-regulation is linked to a cell cycle effect after pimasertib treatment, mRNA levels of RRM1 and RRM2 were analysed 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 down-regulation 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 (Fig S2C). Importantly, pre-treatment 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 cycloheximide, followed by 4 hour treatment with pimasertib and this did not affect pimasertib-induced downregulation of
RRM1 on reducing RRM1 protein (Fig 2E). The same result was found with PANC-1 cells (Fig S3A).

**Pimasertib induces MDM2-mediated polyubiquitination and degradation of RRM1**

Our data suggest that pimasertib sensitizes pancreatic cancer cells to gemcitabine by reducing protein expression of RRM1. However, there may be other mechanisms by which pimasertib 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 pre-treated for 1 hour with MG132, followed or not by 4 hour treatment with pimasertib and subjected to immunoblotting with an antibody against Lys-48. Pre-treatment with MG132 produced a ladder of bands at high molecular weights whose intensity increased after pimasertib treatment in BxPC-3 and PANC-1 cells, suggesting that pimasertib enhances RRM1 poly-ubiquitination 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 down-regulation induced by pimasertib in BxPC-3 cells, compared to cells transfected with scrambled (Fig 3B). Next, to determine if RRM1 can interact with MDM2, co-immunoprecipitation was performed. Endogenous RRM1 bound to MDM2, and there was increased RRM1 binding after treatment with pimasertib.
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 pimasertib on RRM1 stability by inhibiting its binding with MDM2. Addition of 1µM nutlin-3 to pimasertib on BxPC-3 cells (mutant P53 cells) inhibited RRM1 down-regulation induced by pimasertib (Fig 3D), indicating that nutlin-3 blocks MDM2 association with RRM1. Treatment with P53 siRNA did not affect the down-regulation of RRM1 induced by pimasertib suggesting that this effect is not dependent on P53 (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 activation by pimasertib, BxPC-3 and PANC-1 cells were treated for 4 hours with pimasertib, GDC-0942, a PI3 kinase inhibitor, and a combination of pimasertib with GDC-0942. The addition of the PI3 kinase inhibitor impaired RRM1 degradation induced by pimasertib (Fig 4A). The same effect was observed in SUIT-2 cells (Fig. S4B). It is important to note that pimasertib increased AKT phosphorylation as shown by the p-AKT/t-AKT ratio (Figure 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 what has been found in previous studies, BxPC-3 cells were treated with specific siRNA for RRM1 or scrambled (Fig 5A). After 72h, cells were harvested and treated for 48h 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 pimasertib with gemcitabine induced tumor growth delay in an orthotopic model of pancreatic cancer

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

To determine whether pimasertib 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/BL6 black female mice. A 5-days treatment cycle was performed. Mice were treated with vehicle, single agent gemcitabine intraperitoneally 80mg/kg (twice/week), single agent pimasertib via oral gavage at 5mg/kg
(daily) or with pimasertib 5mg/kg (daily) followed 4 hours later by gemcitabine 80mg/kg (twice/week). After two treatment cycles, mice were sacrificed, tumor excised, weighed and sectioned for histopathologic analysis. Mice treated with single-agent pimasertib or gemcitabine had no significant difference in tumor weight compared to the vehicle cohort. In contrast, combination treatment showed tumor sizes significantly lower than treatment with either gemcitabine (*P< 0.05) or pimasertib alone (**P< 0.01). Representative gross images of tumor are shown (Fig 6B). The effect observed was due to lack of growth, as expected, since 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 pimasertib 15mg/kg to gemcitabine 80mg/kg, also significantly reduced tumor weight compared to gemcitabine alone (**P< 0.01) and pimasertib alone (*P<0.05) in TB32048 xenograft mice (Fig S5A). Statistical analysis was performed using the Student’s t test.

Immunohistochemical 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 (CC3). An increase of the percentage of CC3 positive cells and a decrease of ki67 staining was observed in mice treated with pimasertib/gemcitabine regimen compared to vehicle. Specific target inhibition was seen in tumours 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 Figure 6D. Quantification of RRM1, P-ERK and P-AKT staining in tumor tissues treated was performed (Fig 6E).

Discussion

PDAC is a malignancy which 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). Additionally, gemcitabine inactivates RRM1 through covalent binding, thus reducing the amount of dNTP pools available for new DNA synthesis and repair (9). The documented, though 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 PDAC patients treated with gemcitabine. The alteration of chemosensitivity by agents which 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 to 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 two studies in advanced NSCLC have shown that RRM1 expression is inversely correlated with patient’s response to gemcitabine-based therapies (39, 40). Furthermore, high expression of RRM1 is associated with worse survival after gemcitabine treatment in pancreatic cancer patients, suggesting a major role for RRM1 in intrinsic resistance to gemcitabine (41). Several gemcitabine-based combinations have been evaluated in clinical trials and resulted in improved antitumor activity compared to monotherapy treatment (4) (5).

Genetic alterations of the RAS-RAF-MEK-ERK signalling pathway are frequently found in PDAC, particularly, mutation of the K-RAS gene, which contributes to tumor growth and chemoresistance (42); Inhibition of MEK1/2 has become an attractive target in human cancers characterized by aberrantly activated MAPK signalling 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 which 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 (43). Pimasertib (AS703026 or MSC1936369B) 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. (23) (22). 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, antagonising gemcitabine activity, which is S-phase dependent (44). 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 RRM1 downregulation occurs through a post-translational modification. Down-regulation 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 poly-ubiquitinated and identified RNF2 and Bmi1 E3 ubiquitin ligases to be involved in RRM1 degradation by the proteasome (45). Our study showed enhancement of RRM1 poly-ubiquitination through lys48-mediated linkage upon pimasertib treatment.

MDM2 is an E3 ubiquitin ligase that targets P53 for proteasomal degradation (46). 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 down-regulation 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 (47). 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 phosphatidylinositol 3-kinase (PI3K)-AKT signalling 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 (48). Based on our finding, 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.

In accordance with previous published data we confirmed that a correlation exists between RRM1 levels and resistance to gemcitabine (41, 45). Gemcitabine-induced apoptosis was increased and proliferation reduced upon depletion of RRM1 with specific siRNA, confirming its key role in gemcitabine resistance.

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 PDAC patients 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 impacted 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 (Van Cutsem E, et al. J Clin Oncol 33, 2015 [suppl 3; abstr 344]) (49), 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.
References

Figure Legends

Figure 1. The MEK1/2 inhibitor pimasertib sensitizes PDAC cell lines to gemcitabine-induced cytotoxicity. A. Effect of pimasertib treatment on ERK signalling. PANC-1 and BxPC-3 cells were treated with increasing concentrations (0.1µM, 0.5µM, 1µM) of pimasertib for 24 hours or with 1µM pimasertib and extracted at different time points (30min to 48h). Total lysates were analysed by immunoblotting with the indicated antibodies and calnexin was used as loading control. Table 1. The MTT assay was used to assess the effect of a 4-hour pre-treatment with 500nM pimasertib followed by 48h exposure with increasing concentrations of gemcitabine (5-25-50nM) or simultaneous treatment of 500nM pimasertib with increasing concentrations of gemcitabine (5-25-50nM) for 48 hours, on the growth of PANC-1 and BxPC-3 cells. Combination Indices (CI) describing drug combinations were calculated from the mean fraction affected 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 48h treatment with 500nM pimasertib, 50nM gemcitabine and 4h pre-treatment with 500nM pimasertib followed by 48h treatment with 50nM gemcitabine. Results are presented as fold increase to untreated sample and are shown as mean ± SD (n=3). Stars indicate a significant increase in cell death when gemcitabine was combined to pimasertib in PANC-1 (***, P<0.001) and BxPC-3 (***, P<0.001) cells (one-way Anova). C. Effects of MEK1 and MEK2 knock-down on gemcitabine sensitivity. BxPC-3 cells were transfected with a specific siRNA targeting MEK1, MEK2 or non-targeting siRNA (scrambled) for 72h. Transfected cells were treated with 100nM gemcitabine and cell viability was measured by MTT assay 24 hours later. Each experiment was repeated three times and results are presented as mean ± SD (***, P<0.001).
**Figure 2.** MEK inhibition reduces RRM1 protein levels through a post-translational modification. **A.** PANC-1, BxPC-3, SUIT-2 and MIAPaCa-2 Cells were treated for 24 hours with 1µM pimasertib, 50nM gemcitabine alone or in combination with 1µM pimasertib. Immunoblotting analysis was performed on whole-cell lysates. Calnexin was used as 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 analysed by immunoblotting with the indicated antibodies and calnexin was used as loading control. **C.** RNA extraction was performed in BxPC-3 cells after 24h 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. **D.** PANC-1 and BxPC-3 cells, were treated with 1µM pimasertib and extracted at different time points (1h to 4h). Total lysates were analysed by immunoblotting with the indicated antibodies and calnexin was used as loading control. Results are representative of three independent experiments. **E.** BxPC-3 cells were pre-treated with 1µM of proteasome inhibitor MG132 for 1 hour followed by a 4-hour treatment with 1µM pimasertib or pre-treated for 1 hour with 100 µg/ml cycloheximide followed by 24 hour treatment with 1µM pimasertib. Protein lysates were analysed by immunoblotting analysis. Results are representative of two independent experiments.

**Figure 3.** Pimasertib induces MDM2-mediated ubiquitination and degradation of RRM1. **A.** PANC-1 and BxPC-3 cells were pre-treated for 1 hour with 1µM MG132 followed by 1µM pimasertib for 4 hours. Immunoprecipitation was performed using anti-RRM1 antibody or anti-IgG antibody, and immunoblotted with anti-RRM1 and anti-K48 antibodies. Inputs
lysates were blotted with RRM1 antibody. Results are representative of three independent experiments. **B.** BxPC-3 cells were treated with specific RNAi targeting MDM2 (50nM) or scrambled for 72 hours, followed by 4h treatment with 1µM pimasertib. Immunoblotting analysis was performed on whole cell lysates with the indicated antibodies. **C.** BxPC-3 cells were pre-treated for 1h with 1µM MG132 followed or not by 4 hour treatment with 1µM 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 co-treated with 1µM nutlin-3 and 1µM pimasertib for 24 hours and protein lysates were analysed by immunoblotting with the indicated antibodies. Calnexin was used as loading control. Results are representative of two independent experiments.

**Figure 4.** RRM1 degradation induced by MEK inhibition occurs through activation of AKT. **A.** BxPC-3 and PANC-1 cells were treated for 4 hours with 1µM of PI3K inhibitor GDC-0941 together with 1µM pimasertib and whole cell lysates were immunoblotted using the indicated antibodies. β-actin was used as loading control. **B.** Protein levels of P-AKT and T-AKT obtained from three independent experiments were quantified using ImageJ software and plotted as p-AKT/total AKT ratio.

**Figure 5.** siRNA knockdown of RRM1 increases gemcitabine sensitivity. **A.** BxPC-3 cells were transfected with 50nM siRNA targeting RRM1 or scrambled for 72 hours and immunoblotted with the indicated antibodies. Calnexin was using as loading control. **B.** Scrambled and RRM1 transfected cells were treated with 100nM gemcitabine for 48 hours after which MTT proliferation assay was performed. **C.** Caspase 3/7 activity was used to detect apoptosis after 48h treatment with 100nM gemcitabine in RRM1 transfected cells and cells transfected with scrambled. Results are presented as fold increase to
untreated sample and are shown as mean ± SD (n=3). Experiments were repeated three times. Stars indicate a significant increase in inhibition of proliferation (***, P<0.001) and cell death (**, P<0.01) after gemcitabine treatment in BxPC-3 cells transfected with RRM1 siRNA compared to cell transfected with scrambled.

Figure 6. The combination of pimasertib with gemcitabine induces tumor growth delay.

A. TB32048 mouse cells were treated for 4 and 24 hours with 1 µM pimasertib. Total cell lysates were immunoblotted using the indicated antibodies and calnexin was used as loading control. The 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, 5mg/kg pimasertib (daily) by oral gavage, gemcitabine 80mg/kg (twice/week) intraperitonally or a sequential combination of pimasertib 5mg/kg followed by gemcitabine 80mg/kg. Gross images from representative excised tumors are shown at the bottom. A schematic of the schedule regimen utilized in the orthotopic model is illustrated. After sacrifice, tumor weight was measured. Student’s t test was used to determine the statistical significance of differences in tumor weight (***P<0.01 and *P<0.05). C. Tumor volumes were measured by ultrasound analysis before and after treatment. One-way Anova was performed for statistical analysis (***P<0.001). D. Immunohistochemistry (IHC) staining from tumors treated with the indicated drugs and vehicle. Paraformaldehyde fixed paraffin sections were incubated with anti-ki67, anti-CC3, 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 are plotted as mean ± SD. One-way Anova was performed for statistical analysis (**P<0.01, ***P<0.001).
Figure 1A

**PANC-1**

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**PANC-1**

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**BxPC-3**

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TABLE 1

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Figure 2A
Figure 2B

[Image: Western blot analysis showing protein expression levels for RRM1, MEK1, MEK2, and CALNEXIN in PANC-1 and BxPC-3 cells treated with scrambled or MEK1 siRNA.]
Figure 2C
Figure 2D
Figure 2E
Figure 3A

![Image of a figure with Western blot results comparing PANC-1 and BxPC-3 cell lines treated with Pimasertib and MG132. The figure shows the effect on the expression of RRM1, K-48, and CALNEXIN proteins.](image-url)
Figure 3B

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Figure 3D

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RRM1

CALNEXIN
Figure 5A

BxPC-3

CONTROL  SCRAMBLED  RRM1 siRNA

RRM1

CALNEXIN
Figure 5C

[Graph showing cleaved caspase 3-7 activity for BxPC-3 cells treated with control or gemcitabine, with bars for scrambled and RRM1 siRNA groups.]
Figure 6A

TB32048

PIMASERTIB 1uM

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P-AKT/ACT ratio

- UNTREATED
- PIMASERTIB 1uM
Figure 6B

The figure shows a bar graph and images of tumor weights. The x-axis represents different treatments: vehicle, pimasertib, gemcitabine, and pimasertib/gemcitabine. The y-axis represents tumor weight in grams. The graph includes multiple data points and error bars, indicating variability in tumor weight across different treatments.

Key points:
- **Vehicle**
- **Pimasertib**
- **Gemcitabine**
- **Pimasertib/Gemcitabine**
- **GEMCITABINE 80mg/kg 2/week - ip**
- **PIMASERTIB 5mg/kg 5D- po 4h later**

The graph includes statistical significance markers (e.g., **, *) indicating differences in tumor weight between treatments.
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FIGURE 6D

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Figure 6E

**RRM1**

**P-ERK**

**P-AKT**
Clinical Cancer Research

THE MEK1/2 INHIBITOR PIMASERTIB ENHANCES GEMCITABINE EFFICACY IN PANCREATIC CANCER MODELS BY ALTERING PROTEIN LEVELS OF RIBONUCLEOTIDE REDUCTASE SUBUNIT-1 (RRM1)

Francesca Vena, Eleonora Li Causi, Manuel Rodriguez-Justo, et al.

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